SAMHD1 ACTIVITY UNDERLIES IFN RESPONSES AND SEX-DEPENDENT DIFFERENCES IN MACROPHAGE INFECTION BY HIV-1

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Human Immunodeficiency Virus type 1 (HIV-1) targets immune cells, including CD4+ T cells, macrophages, and dendritic cells (DC). Macrophages are critically important for their contributions to viral propagation and inflammation in vivo during infection, though the current understanding of host-pathogen interactions in this cell type remains deeply inadequate. A better understanding of how HIV-1 infects macrophages despite facing numerous cellular defenses may inform therapeutic approaches aimed at preventing HIV-1 infection and its sequelae. A major component of the anti-HIV-1 defense is SAM domain and HD domain-containing protein 1 (SAMHD1), a phosphohydrolase responsible for restricting the generation of double-stranded viral DNA (dsDNA) from a single-stranded RNA (ssRNA) genome. It functions by converting deoxynucleoside triphosphates (dNTP) to deoxynucleosides (dN) in noncycling cells, a process dependent on tetramerization that is inhibited through threonine-592 (T592) phoshphorylation. We discovered that SAMHD1 activity is a critical determinant of HIV-1 infection in macrophages, and that despite its abundant expression, can exist in an inactive state (phosphor-T592) allowing macrophages to be highly susceptible to HIV-1. We found that biological sex is a major determinant of macrophage infectivity with HIV-1, and that the relative susceptibility of male- versus female-derived macrophages hinges exclusively on SAMHD1 activity. Furthermore, we have established SAMHD1 as the principal effector through which diverse interferons (IFN) act in macrophages, and that

activation of SAMHD1 through IFN signaling establishes a state of HIV-1 resistance. We find that cyclin-dependent kinase 1 (CDK1) is the major determinant of IFN-induced SAMHD1 activation, and that depletion of CDK1 through siRNA alone drives SAMHD1 activation in MDM. Interestingly, a variety of FDA-approved tyrosine kinase inhibitors (TKI) phenocopy the effects of IFNs, and their activity depends on their ability to activate SAMHD1. Furthermore, we find that macrophage infection with HIV-1 induces a similar state of protection from subsequent challenge, which correlates with activation of SAMHD1. Interestingly, this protection does not appear to involve the production of IFNs. Using phosphoproteomic and RNA-sequencing approaches, we have begun to unravel the pathways responsible for SAMHD1 activation resulting from HIV-1 sensing, IFN stimulation, and the use of TKIs, which exhibit overlapping but distinct mechanisms of action.

TABLE OF CONTENTS

LIST OF FIGURES

Figures

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CHAPTER 1

INTRODUCTION

Human immunodeficiency virus type 1: past and present

Acquired immunodeficiency syndrome (AIDS) was first recognized in patients in the United States as early as 1981, under the guise of patients infected with a rare pneumonia caused by *Pneumocystis carinii* or those presenting with a rare form of cancer, Kaposi's Sarcoma [1]. The appearance of AIDS was inopportune: the advent of effective antimicrobials precipitated a general sense of complacency toward infectious microbes, and many believed that the war on microorganisms such as bacteria, fungi, and viruses had been won. A prevailing sense that microbes, at least in industrialized countries, no longer posed a threat to humanity, hung over science and medicine [2, 3]. Retroviruses themselves were not believed to cause disease in humans, as efforts to find retroviruses, much less attribute them to diseases had proved fruitless. The blossoming field of retrovirology as we know it today withered while a global pandemic loomed [4].

It had been long hypothesized that certain viruses had the ability to cause tumors, termed oncogenic viruses. However little data to support the existence of human oncogenic retroviruses was available until the early 1980s [5]. A few laboratories persisted in their efforts to characterize these esoteric pathogens, namely those of Howard Temin and David Baltimore. The stage was set in 1970 with the co-discovery of reverse transcriptase (RT) as a component of Rous Sarcoma Virus (RSV) by Temin and Baltimore [6-8], who had been tenacious advocates for the existence of oncogenic viruses. This breakthrough, profound in its challenge of the 'central dogma', set the stage for the discovery of the first leukemogenic virus, human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) [5, 9]. By 1979, it was clear that viruses were not only capable of causing human cancers, but that they possessed the ability to pass genetic

information from RNA to DNA. It would not be long before the AIDS epidemic made landfall, and while the causative agent remained elusive, these discoveries laid the critical groundwork for the breakthrough discovery of HIV [10, 11].

By the mid-1980s, AIDS was widely recognized as a persistent disease with a significant period of clinical dormancy from exposure to immunosuppression. However few clues existed regarding potential causes of AIDS[10, 12]. One common and major theme permeated AIDS cases: patients experiencing profound immunosuppression shared a precipitous decline in CD4+ T cell counts [13-15]. HTLV immediately emerged as a prime suspect. It was known that HTLV was transmitted through blood and sexual activity, and it had been previously shown that lymphotropic viruses were capable of causing AIDS-like syndromes in various animal models [5, 9]. The theory that a retrovirus was responsible for AIDS proved correct, but not until 1983 would it be recognized that a new virus, distinct from HTLV, was responsible for AIDS [11, 16].

Characterization of that virus followed, and by 1984 it was clear that this newly discovered pathogen was indeed a lentiretrovirus, and was initially characterized as lymphadenopathy-associated virus (LAV) or HTLV-III [17, 18]. In May 1986, the virus was reclassified as Human Immunodeficiency Virus (HIV). Isolation of another strain in West Africa, HIV-2, from patients exhibiting similar symptoms to patients in the United States supported a causal relationship between these viruses and AIDS-defining illnesses [5]. The establishment of the HIV-AIDS relationship paved the way for novel therapies, mainly those that target enzymes required for viral replication, including viral reverse transcriptase and integrase [19, 20]. As HIV therapies became mainstream and proved efficacious at preventing virus transmission and blunting AIDS-defining illness, a new

era was entered in the fight against HIV. By 1995, patients receiving highly active antiretroviral therapy (now termed combined antiretroviral therapy, HAART or cART) were experiencing what many hoped might be a cure [21].

The current state of the HIV-1 pandemic is one of both promise and uncertainty. The hope for a cure, spurred on by highly effective ART that both brought virus to undetectable levels in patients infected with HIV-1, proved premature. Indeed, patients living with HIV-1 experience CD4+ T cell rebound and return to near-normal health following ART. However cessation of therapy has been demonstrated to result in viral rebound and subsequent disease [21-24]. The viral sanctuary, termed the latent HIV-1 reservoir, had been identified in long-lived memory CD4+ T cells [25]. This reservoir is the single greatest barrier to achieving HIV-1 cure, and is responsible for the requirement of life-long ART. Strategies aimed at achieving so-called latency 'reversal', as well as identification of other potential cells or tissue sites responsible for harboring latent HIV-1 constitute the current state of cure efforts [26, 27].

Immune cells and HIV-1 infection

HIV-1 exhibits tropism for three major immune cell types: CD4+ T cells, macrophages, and dendritic cells [28]. A hallmark of HIV-1 infection and progression to AIDS is the depletion of T lymphocytes, and it is well understood that progressive loss of CD4+ T cells is responsible for the vast majority of immunodeficiency associated with untreated HIV-1 infection [15]. Although myeloid cells are bona fide targets for HIV-1 infection, their role in mediating immunodeficiency and progression to AIDS is not well understood [28]. Indeed, macrophages can support HIV-1 infection and persistence in the

complete absence of CD4+ T cells despite long-term ART, a finding that indicates myeloid cells are an important source of virus replication and contribute functionally to the latent reservoir [29, 30]. Though some evidence exists to support a role for these cells in HIV-1 persistence during therapy, whether these cells harbor truly latent provirus remains an important question in the field [31]. Nevertheless, myeloid cells constitute an important and arguably neglected area of research in the field of HIV-1.

Macrophages as key players in HIV-1 pathogenesis

Macrophages are present in a variety of tissue sites, and their capacity for infection with HIV-1 poses a number of distinct clinical problems. Perivascular macrophages and microglia, present in the brain, are considered major drivers of an important clinical phenomenon known as HIV associated neurocognitive disease (HAND) [32-34]. HAND affects HIV-1-infected individuals in the presence or absence of ART, though the effects are strongly attenuated when viral suppression is achieved [34]. Two major mechanisms for HAND have been proposed: either low-level, ongoing virus replication in sanctuary tissue sites or, alternatively, viral products carried over from untreated HIV-1 infection perpetuate a state of chronic inflammation involving immune cells as well as astrocytes [33]. While virus sequences isolated from the cerebral spinal fluid (CNS) of ART-suppressed patients has been shown to exhibit macrophage tropism, it is still unknown whether these cells represent a functional HIV-1 reservoir [35]. In either case, infection of macrophages and macrophage-like cells is the predominant theory regarding HAND etiology, and these cells are likely to play a significant role in the systemic inflammation observed in chronically-infected patients.

SAMHD1: at the nexus of cell cycle control and virus restriction

Macrophages represent a major cellular target for HIV-1 that, unlike activated CD4+ T cells, are postmitotic and present unique barriers to infection that distinguish them from lymphocytes [36, 37]. For example, macrophages express low levels of the HIV-1 receptor and co-receptor, CD4 and CCR5, predisposing them to low rates of infectivity when compared to CD4+ T cells [38]. Furthermore, the nature of mature macrophages as terminally differentiated cells presents a major barrier to infection once a virus has entered the cell. Because macrophages are nondividing, they maintain limiting concentrations of dNTPs which are required for cellular DNA replication but are also necessary for generation of dsDNA from the ssRNA HIV-1 genome [39]. The mechanisms governing dNTP homeostasis are well known, and in many nondividing cells this process is controlled through the cellular protein SAM domain and HD domain-containing protein 1 [40]. SAMHD1 is a triphosphohydrolase that limits dNTP pools by catalyzing their conversion to inorganic phosphates and deoxynucleosides (dN) [41]. SAMHD1 is highly expressed in macrophages and other myeloid-lineage cells, and its ability to restrict both cell division and virus infection hinges on its capacity for dNTP hydrolysis [40]. It is a notable driver of autoimmune diseases, including systemic lupus erythematosus and Aicardi-Goutieres syndrome, known generally as interferonopathies due to a characteristic overproduction of interferons and subsequent chronic inflammation [42-44].

The ability of SAMHD1 to restrict HIV-1 replication is largely dependent on two major checkpoints: it is controlled at both the level of transcription and at the level of phosphorylation. In dividing cells such as activated CD4+ T cells, SAMHD1 expression

is low and remains largely in a hyperphosphorylated, inactive state [45, 46]. In stark contrast to CD4+ T cells, macrophages and other nondividing immune cells express high levels of SAMHD1 that remains in a hypophosphorylated, active state [45, 47]. Unsurprisingly, SAMHD1 phosphorylation is controlled by a family of cyclin-dependent kinases (CDK), which in response to mitogenic stimuli, quickly phosphorylate and inactivate its catalytic activity [48]. Thus, cells expressing SAMHD1 can rapidly modulate its activity to suit the dNTP requirements of the cell. This is abundantly clear in CD4+ T cells, which rapidly phosphorylate SAMHD1 in response to CD3/CD28 stimulation. Thus, SAMHD1 is not considered a major barrier to infection in cycling cells expressing an abundance of CDKs that are known to act on SAMHD1 [45-47].

The nature of SAMHD1 in macrophages and the ability of its activity to be modulated has been the subject of recent intense inquiry. We have recently shown that the ability of macrophages to be infected by HIV-1 can be predicted by the degree of SAMHD1 present in its inactive, phosphorylated state, as outline in Chapter 2 and Chapter 3. While macrophages largely resist infection with HIV-1 due to the expression of SAMHD1, enzymatic activity appears to be governed by a number of CDKs, which are in turn controlled by cell signaling pathways that can be targeted through kinase inhibition. The precise pathways responsible for modulation of SAMHD1 activity in macrophages remains unknown, and whether phosphatases are key to the rapid induction of SAMHD1 activity remains to be elucidated. Further studies aimed at delineating the relevant pathways may provide insight not only into HIV-1 restriction, but may also prove relevant to other disease states which are known to be caused by aberrant control of SAMHD1.

Dissertation summary

The work included in this dissertation uncovers a role for SAMHD1 in response to numerous stimuli, and investigates this host restriction factor as the major modulator of infection in macrophages. Utilizing primary human monocyte derived macrophages (MDM), we have demonstrated that the major antiviral activity of various IFNs hinges on their ability to activate SAMHD1, and that IFNs act largely through the induction of CDK1. We show that pharmacologic manipulation of SAMHD1 by FDA-approved tyrosine kinase inhibitors (TKI) can induce a state of potent HIV-1 restriction in macrophages that is strictly dependent SAMHD1 activation. Furthermore, we have demonstrated sex bias in HIV-1 infection of MDM, and have described SAMHD1 as a novel regulator of sex-based immune differences. Work aimed at elucidating the precise signaling events that lead to changes in SAMHD1 activity is ongoing, as are further studies characterizing how macrophages respond to infection by entering a state of HIV-1 resistance.

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CHAPTER 2

SEX INFLUENCES SAMHD1 ACTIVITY AND SUSCEPTIBILITY TO HIV-1 IN PRIMARY HUMAN MACROPHAGES

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Abstract

While it is known that host sex affects HIV-1 viremia and influences the severity of HIV-1 associated neurocognitive disease, a cellular or molecular basis for these findings remains elusive. Macrophages are major targets for HIV-1 and contribute to viral propagation in vivo. In the present study, we aimed to address whether sex affects HIV-1 infectivity of primary human macrophages and CD4+ T cells in vitro. We find that donor sex influences infectivity of monocyte-derived macrophages: macrophages derived from healthy female donors were less susceptible to HIV-1 infection than those derived from healthy males. This sex-dependent difference was independent of viral entry, originated prior to viral integration, and was not observed when in CD4+ T cells from the same donors. We report that differential regulation of the host restriction factor SAMHD1 is the main factor governing the sex-dependent difference in infectivity. The sex-dependent differences in SAMHD1-mediated viral restriction were abolished by Vpx-mediated depletion of SAMHD1 or by addition of exogenous deoxynucleosides. We conclude that SAMHD1 is an essential modulator of infectivity in a sex-dependent manner in macrophages but not in CD4+ T cells, and constitutes a novel component of sex-dependent differences in innate immune control of viral infection.

Introduction

Sex is an important biological variable with regard to susceptibility and natural history of many diseases including HIV-1, though the cellular and molecular basis of these observations remains largely unclear. Women constitute the majority of adults living with HIV-1, with an estimated 17.8 million women infected, compared to 16.7

million men worldwide according to the 2016 UNAIDS data. However, participants in clinical research exploring HIV-1 pathogenesis and management overwhelmingly skew male. Under-representation of female participants in HIV-1 research, paired with the implicit assumption that observations gleaned from overwhelmingly male study populations can be extrapolated to females, potentially compromises both the basic and clinical understanding of the HIV-1 epidemic.

Sex differences in acute HIV-1 infection, progression to acquired immunodeficiency syndrome (AIDS), and development of HIV-associated neurocognitive disease (HAND) have been examined. Cross-sectional and longitudinal cohort studies have described sex differences in plasma viral loads and CD4+ T cell counts among new seroconverters and those with chronic HIV-1 infection alike [1-4]. Indeed, multiple lines of evidence have shown that HIV-1-infected women maintain lower levels of plasma viremia and higher CD4+ T cell counts than men in the absence of antiretroviral therapy (ART)[5]. While viral loads in acute infection display sex-dependent variability, progression to AIDS between men and women occurs at similar rates [5]. While a variety of geographic and socioeconomic issues are critically important factors affecting sex-dependent differences, especially in sub-Saharan Africa where rates of new HIV-1 infections are highest, little is known about the contribution of human biology to sex disparities [6].

A precedent for sex-dependent differences in myeloid cells was established with the discovery of sex bias in plasmacytoid dendritic cell (pDC)-mediated responses to HIV-1. Female-derived pDCs exhibit more robust interferon (IFN) responses to infection in a TLR7-dependent manner [7]. Exposure to several estrogen steroid hormones in vitro

results in induction of IFN- and interleukin (IL)-driven antiviral responses [8-10]. IFNs themselves have been shown to be important players in sites of transmission such as the female reproductive tract, indicating differential expression of interferon-stimulated genes (ISGs) may play an important role in sex differences in viral and bacterial infections [11, 12]. Though these studies provide novel insights into sex-dependent differences in virus transmission, the cellular and molecular mechanisms underlying these differences remain unknown.

In the present study, we sought to elucidate whether intrinsic differences in macrophage and CD4+ T cell susceptibility to HIV-1 exist between males and females in vitro. We report that female-derived macrophages potently resist infection with HIV-1, and that their capacity for HIV-1 restriction relative to males is independent of virus and is effected prior to integration. We show that SAMHD1, a well-known anti-HIV-1 restriction factor expressed in immune cells, exhibits sex-dependent regulation in macrophages but not in CD4+ T cells and largely explains the infectivity differences. Thus, the ability of SAMHD1 to restrict viral infection is influenced by sex, and may inform future studies aimed at understanding the molecular basis for sex differences in HIV-1 pathogenesis.

Results

Sex influences macrophage infection with HIV-1

To investigate the role of sex in HIV-1 infection, healthy donors were recruited in male/female pairs. Monocyte-derived macrophages (MDM) were infected with two replication-competent virus clones, HIV-1-NL-4-3-BaL-IRES-HSA and HIV-1-NL-4-3-

AD8, both R5-tropic viruses (Figure S2.1). HIV-1-NL-4-3-BaL-IRES-HSA expresses CD24, also known as heat stable antigen (HSA), and infection is analyzed by the expression of HSA the surface of infected cells[13]. HIV-1-NL-4-3-AD8 infection is analyzed by staining for HIV-1 p24. Macrophages were detached and analyzed via flow cytometry. Infection levels are expressed as % infected macrophages as determined percentage of HSA+ cells (HIV-1-NL-4-3-BaL-IRES-HSA) or p24+ cells (HIV-1-NL-4-3-AD8). We observed a wide range of donor susceptibility to infection (HIV-1-NL-4-3-BaL-IRES-HSA: 1.42-27.90% (males), 0.28-5.62% (females); HIV-1-NL-4-3-AD8: 1.42-12.57% (males), 0.30-4.38% (females)) (Figure 2.1). If infections in male-derived macrophages in each donor pair were normalized to 100%, infections of female macrophages reached infection levels, on average, of 25% (HIV-1-NL-4-3-BaL-IRES-HSA) and 37% (HIV-1-NL-4-3-AD8) (Figure 2.1).

Sex-dependent differences in HIV-1 infection occur early, are independent of virus entry, and represent a preintegration block in female-derived MDM

To determine whether the observed sex-dependent difference in infectivity required multiple rounds of replication, MDM were generated from six healthy donors and infected with HIV-1-NL-4-3-AD8. Infection was tracked over a 14-day time course and analyzed at 2, 6, 10, and 14 days postinfection. Significant sex-dependent differences in infectivity were observed 2 days postinfection, and differences were compounded at the later time points (Figure 2.2). These results suggest sex-dependent differences are due

to an intrinsic difference in susceptibility that renders female-derived macrophages less permissive to initial infection independent of replication [14].

We next sought to ascertain whether the route of virus entry can explain the observed sex-dependent differences in HIV-1 infectivity. To this end, we generated replication-defective HIV-1 viruses pseudotyped with vesticular stomatitis virus glycoprotein (HIV-1−ΔEnv-GFP/VSVG). HIV-1−ΔEnv-GFP/VSVG contains a frameshift mutation in the envelope glycoprotein gene and is capable of only a single round of infection. Importantly, VSVG utilizes the low-density lipoprotein receptor, which shunts viruses towards an endocytic pathway that is independent of gp160-receptor/co-receptor interactions [15]. Infection of paired male and female healthy donor MDM revealed differences in infectivity that mirrored those observed during infection with viruses encoding a natural HIV-1 envelope glycoprotein (Figure 2.2). Setting infection of male macrophages in each donor pair to 100%, females exhibited, on average, infection levels that reached 39% of those observed in paired male counterparts (Figure 2.2). Analysis of the HIV-1 receptor/co-receptors CD4 and CCR5 by flow cytometry revealed no differences in cell surface expression, consistent with the notion that the relative intrinsic susceptibility of male MDM to infection occurs at a receptor-independent, postentry step (Figure S2.2).

We next examined total genomic DNA of HIV-1−ΔEnv-GFP/VSVG-infected MDM to quantify integration events in an effort to identify where in the HIV-1 life cycle sex differences in susceptibility to infection occur. Infection in six donor MDM was analyzed by flow cytometry for GFP expression, and on average, 10.6% of male MDM were infected compared to 2.5% of female-derived MDM. Gag-PCR revealed significant

differences between male- and female-derived macrophages, supporting a model wherein female-derived macrophages restrict HIV-1 with greater potency at a preintegration step (Figure 2.3).

Memory CD4+ T cells do not display sex-dependent differences in infectivity

To determine whether observed differences in infectivity were unique to MDM or were also displayed by T-cells from the same donors, CD4+ T cells and MDM were isolated from paired male and female donors cells and were infected ex vivo via spinoculation with HIV-1-NL-4-3-BaL-IRES-HSA, the same viral clone utilized in previous experiments involving MDM infections. Unlike MDM, memory CD4+ T cells failed to exhibit significant sex differences in initial infectivity regardless of MOI, and analysis of infection at the 96-hour time point failed to reveal differences in replication between males and females (Figure 2.3). Similar to our experiments in MDM, we observed no differences in the expression of either CD4 or CCR5 (Figure 2.3). These experiments support a myeloid cell type-specific, sex-dependent difference in infectivity with HIV-1.

Sex-dependent differences in MDM infectivity are SAMHD1-dependent

SAMHD1 has been shown to potently restrict HIV-1 at a preintegration step through inhibition of reverse transcription [16-18]. SAMHD1 is a deoxynucleoside triphosphate (dNTP) triphosphohydrolase, and its activity as an HIV-1 restriction factor is inhibited by threonine-592 phosphorylation by destabilizing the tetramer required for its catalytic activity [19-21]. To examine the potential role of SAMHD1 in regulating sex-

related differences, MDM were generated from six donors were lysed on day 7. Whole cell lysates were analyzed by Western Blot for levels of total SAMHD1, SAMHD1-pT592, and β-Actin. Total levels of SAMHD1 did not differ between male- and female-derived MDM. However levels of the active enzyme, as measured by phospho-T592, were present at a significantly higher degree in female-derived MDM (Figure 2.4, two representative donor pairs shown). This represented, on average, a 2-fold increase in relative levels of pSAMHD1 in male- versus female-derived MDM (Figure 2.4).

Sex-dependent differences are overcome through bypass of SAMHD1-mediated restriction

In view of the above results, we hypothesized that enhanced activity of SAMHD1 was responsible for the relative restriction observed in female-derived macrophages. Thus we predicted that addition of an excess of exogenous deoxynucleosides (dN) would ablate the sex-dependent differences. dN 'flooding' has been shown to overcome the SAMHD1-mediated restriction and render macrophages generally more permissive to infection [16]. As shown in our previous report, dN flooding relieved baseline restriction and extinguished sex-based differences in infectivity, enhancing female-derived MDM (Donor A026) infectivity from 0.8% (no dN) to 12.8% (10mM dN) and male-derived MDM (Donor A006) infectivity from 2.9% (no dN) to 11.6% (10mM dN) [22].

To confirm that SAMHD1 is responsible for the differences observed, we utilized the HIV-2 accessory protein Vpx. Vpx targets SAMHD1 for ubiquitination, leading to proteasome-directed proteolysis in the setting of HIV-2 infection [16-18, 23, 24]. Though HIV-1 does not encode Vpx, and therefore does not induce SAMHD1 degradation on its

own, it can be complemented with Vpx through virus-like particle (VLP)-mediated delivery to enhance HIV-1 infection of myeloid cells [18, 25]. In agreement with our experiment utilizing excess dNs to overcome the SAMHD1-mediated restriction, pre-treatment of cells with Vpx(+)VLPs completely reversed the restrictive capacity of MDM (Figure 2.5, two representative donors shown). Importantly, Vpx(+)VLPs effectively eliminated differences in infectivity between three male- and three female-derived MDM (Figure 2.5). These experiments confirm that SAMHD1 is the key antiviral restriction factor responsible for sex-dependent differences in MDM infectivity with HIV-1.

Materials and methods

Generation of monocyte-derived macrophages and isolation
of memory CD4+ T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy-donor whole blood via Lymphoprep density gradient centrifugation (StemCell Technologies) immediately following phlebotomy. Positive selection of CD14+ monocytes was carried out using magnetic bead-based isolation (Miltenyi Biotec). CD14+ monocytes were resuspended in serum-free RPMI 1640 (Invitrogen) supplemented with 100U/mL Penicillin, 100ug/mL Streptomycin, 2mM L-Glutamine (Gibco), seeded at a density of 1.0x10^6 cells/well (12-well plates, Corning) or 5.0x10^5 cells/well (24-well plates, Corning), and incubated for 2 hours at 37 degrees C and 5% CO2 to allow optimal adherence. Serum-free media was aspirated and replaced with macrophage growth media, consisting of RPMI 1640 supplemented with 100U/mL Penicillin, 100ug/mL Streptomycin, 2mM L-Glutamine (Gibco), and 12% Pooled Human Serum (Innovative

Research). CD14+ monocytes were cultured for 7 days in macrophage growth media prior to downstream analysis. Untouched memory CD4+ T cells were isolated via negative isolation from the remaining fraction following CD14+ monocyte isolation (Miltenyi Biotec). Memory CD4+ T cells were cultured for 2-5 days in macrophage growth media prior to infection. Infection was carried out via spinoculation at 2900rpm for 2 hours at 37 degrees C.

Viruses and virus clones

Replication-defective (HIV-1−ΔEnv-GFP) and replication-competent (HIV-1-BaL-IRES-HSA and HIV-1-NL4-3-AD8) were used in this study. Replication-incompetent HIV-1−ΔEnv-GFP clones contain a frameshift mutation in Env, express either GFP or mCherry in place of Nef, and were pseudotyped with Vesicular Stomatitis Virus Glycoprotein (VSVG). The replication-competent, R5 clone HIV-1-NL4-3-BaL-IRES-HSA was a kind gift of Michel J. Tremblay (Universite Laval, Quebec City, Quebec), and expresses a surface-bound CD24 (HSA) from an IRES. The replication-competent, R5 clone HIV-1-NL4-3-AD8 is a derivative of pNL4-3 (Eric O. Freed, NIH AIDS Reagent Program).

Virus generation and infection of MDM

Viruses were generated via calcium phosphate-mediated transfection of HEK293T cell with either HIV-1−ΔEnv-GFP, HIV-1-NL4-3-BaL-IRES-HSA, or HIV-1-NL4-3-AD8 clones. Supernatants were collected 36 hours following transfection and cryopreserved in -80 degrees C until use. Primary MDM were infected in static culture

for 12 hours following 7-day spontaneous differentiation by diluting 250ng of virus as determined by p24-ELISA in 500uL RPMI 1640. Following infection, viral supernatant was removed, MDM were washed twice with serum-free RPMI 1640, and media was replaced with macrophage growth media.

Flow cytometric analysis

Macrophages were detached from tissue culture plates via incubation with Accutase Cell Dissociation Reagent for 2 hours at 37 degrees C. Cells were washed and stained with eFluor450 Viability Dye (eBioscience). To phenotype cells, cells were stained with the following monoclonal antibodies: Fluorescein isothiocyanate-conjugated (FITC)-anti-CD14, allophycocyanin-conjugated (APC)-anti-CD16, APC-anti-CD71, and phycoerythrin-conjugated (PE)-anti-CD86 followed by flow cytometric analysis in a LSRFORTESSA X-20 (BD Bioscience). To assess viral gene expression, HIV-1–ΔEnv-GFP/VSVG-infected cells were analyzed for GFP reporter gene expression. Cells infected with HIV-1-BaL-IRES-HSA were stained for surface expression of CD24 with APC-anti-CD24 (eBioscience). Cells infected with HIV-1-NL4-3-AD8 were permeabilized and fixed with Cytofix/Cytoperm (BD Biosciences) for 30 minutes at 4 degrees C, then washed with Perm/Wash Buffer (BD Biosciences) and stained with a 1:40 dilution of anti-p24 antibody (Beckman Coulter).

Discussion

While CD4+ T cells constitute the major target of HIV-1 and contribute to viral latency and persistence in vivo, mounting evidence supports an important role for

myeloid lineage cells in acute and chronic infection [26-31]. Specifically, macrophages are present in a variety of tissues, and are enriched in mucosal sites of HIV-1 transmission [32, 33]. Additionally, macrophages and other myeloid lineage cells are thought to play a crucial role during chronic infection and contribute to pathogenesis in various anatomical sites, including the brain [34]. Persistent inflammation resulting from virus replication gives rise to HAND, resulting in lasting impairments that linger despite long-term ART [35, 36]. Recently, HIV infection in macrophages and microglia has shown to directly contribute to overproduction of neurodegenerative beta-amyloid protein, providing clues into the molecular mechanisms that contribute to HAND [37]. Despite the depth of knowledge regarding HIV-1 pathogenesis in CD4+ T cells, little is known about the role of macrophages in acute and chronic infection, and even less regarding their contribution to sex-based differences.

SAMHD1 is among the most important restriction factors determining the low permissivity to infection of nondividing cells such as macrophages, dendritic cells, and memory CD4+ T cells [16-18, 38, 39]. The expression of SAMHD1 is an important facet in determining the cellular susceptibility to HIV-1. Activated, cycling CD4+ T cells that express low levels of SAMHD1 are exquisitely sensitive to infection with HIV-1, whereas cells that express high levels of SAMHD1 such as macrophages resist infection [16-18, 40]. An important mechanism of SAMHD1 regulation in cells enriched for SAMHD1 expression is threonine-592 phosphorylation [19-21]. T592 phosphorylation leads to destabilization of the SAMHD1 tetramer which is required for its anti-HIV-1 activity [20, 21]. In activated CD4+ T cells, SAMHD1 phosphorylation is robustly enhanced in order to satisfy the cell's requirement for dNTPs in DNA synthesis [19].

SAMHD1 phosphorylation in macrophages and other noncycling cells is similarly under dynamic control and is dependent on cues that range from growth factor signaling to pathogen recognition [19, 22, 41].While it has been shown that TLR7-dependent sex differences exist in dendritic cell susceptibility and response to HIV-1, a function for differential regulation of SAMHD1 between males and females has not, to our knowledge, been previously reported. In this report, we identify SAMHD1 regulation as a novel molecular mechanism underlying sex differences in infectivity of human macrophages.

The contribution of IFNs to HIV-1 restriction is well established, and multiple downstream mechanisms that block infection from entry to release have been studied and reviewed at length [42]. An important consequence of IFN signaling is the de-phosphorylation and activation of SAMHD1, which presents a potent barrier to infection that can be overcome by SAMHD1 proteolysis or siRNA-mediated silencing [22]. Given that a sex-dependent difference has been observed in IFN production in pDCs in response to TLR signaling, it is formally possible that baseline differences in IFN production contribute to differences in MDM infection with HIV-1 [7]. A type I IFN, IFN epsilon, is constitutively expressed in cells of the female reproductive tract, and its expression is highly induced upon steroid hormone treatment [12]. This finding provides an intriguing avenue for future research into sex-dependent differences in HIV-1 infection, and may potentially play a role in the steady-state protection and SAMHD1 activity female macrophages exhibit ex vivo. The fact that women are disproportionately affected by autoimmune disorders, particularly interferonopathies such as systemic lupus erythematosus, warrants further investigation into differences in immunologic pathways

that govern both susceptibility to viral infections on one hand and chronic inflammatory diseases on the other. The results of the current study demonstrate the importance of understanding sex as a biological variable in HIV-1 infection, and the importance of including research participants of both sexes in clinical and basic research efforts. Furthermore, we establish for the first time SAMHD1 and its regulation as a critical component of sex differences in the control of HIV-1 infection, which may inform future investigations into natural immunity to HIV-1 and predisposition to conditions such as HAND.

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Author contributions

Conceptualization, M.A.S. and V.P.; Methodology, M.A.S. and V.P.; Investigation, M.A.S and V.P.; Writing – Original Draft, M.A.S. and V.P.; Writing – Review & Editing, M.A.S, V.P., A.M.S., and A.B.; Funding Acquisition, M.A.S. and V.P.; Supervision, V.P., A.M.S., and A.B.

Figure 2.1. HIV-1 inefficiently infects female-derived macrophages. (A) Male- (blue) and female-derived (red) MDM infected in pairs with HIV-1-NL-4-3-BaL-IRES-HSA. Macrophages were stained for cell surface CD24 (HSA). Percentage infected macrophages represented as percentage of CD24+ cells. (B) Male- and female-derived MDM infected in pairs with HIV-1-NL-4-3-AD8. Macrophages were permeabilized and stained for intracellular p24. Percentage infected macrophages represented as percentage of p24+ cells. (C) Comparison of female- versus male-derived MDM infected with HIV-1-NL-4-3-BaL-IRES-HSA. Infection of male-derived MDM in each donor pair was set independently to 100% and the percentage of infection in female-derived MDM expressed as a percentage of that observed in the male-derived MDM counterpart. (D) Comparison of female- versus male-derived MDM infected with HIV-1-NL-4-3-AD8. Values calculated as described in (C).

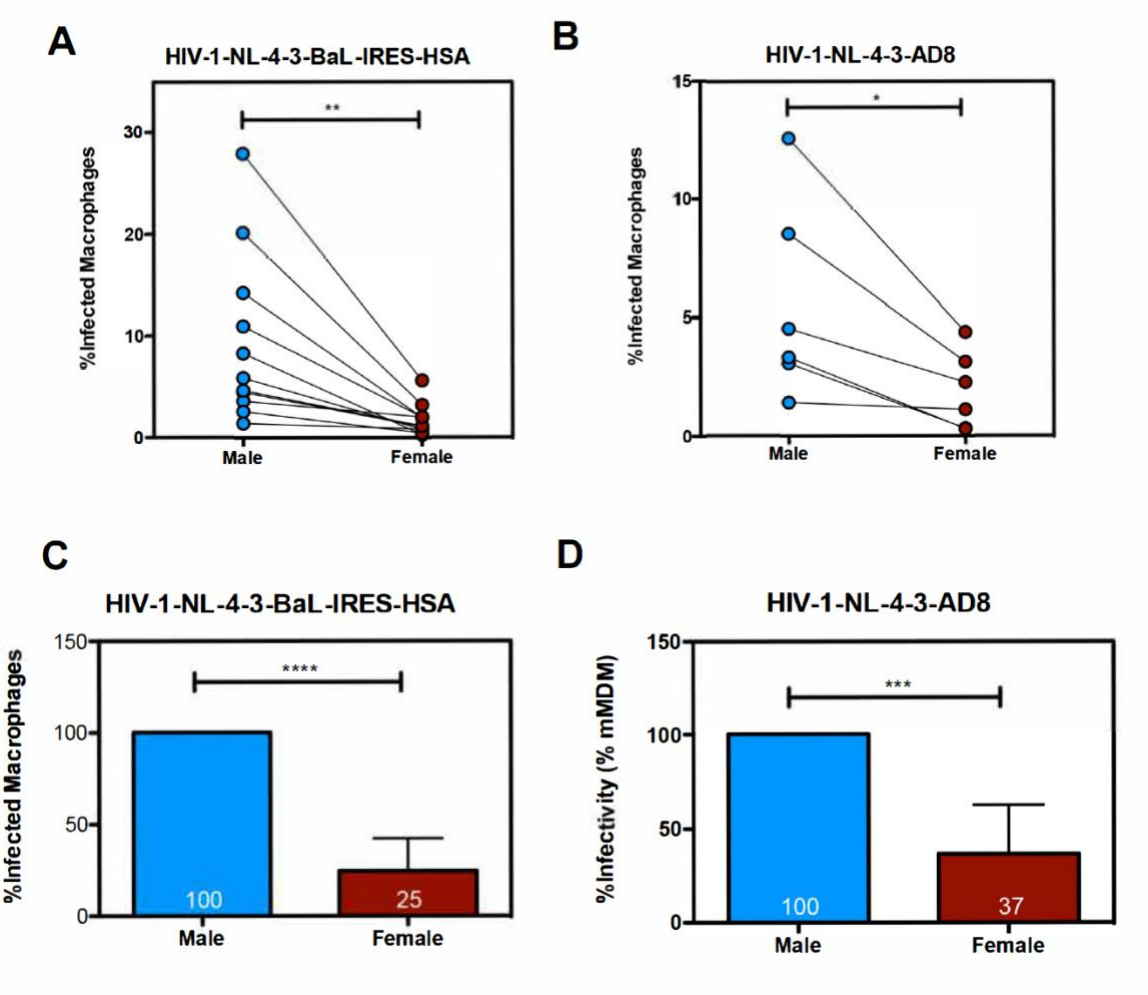
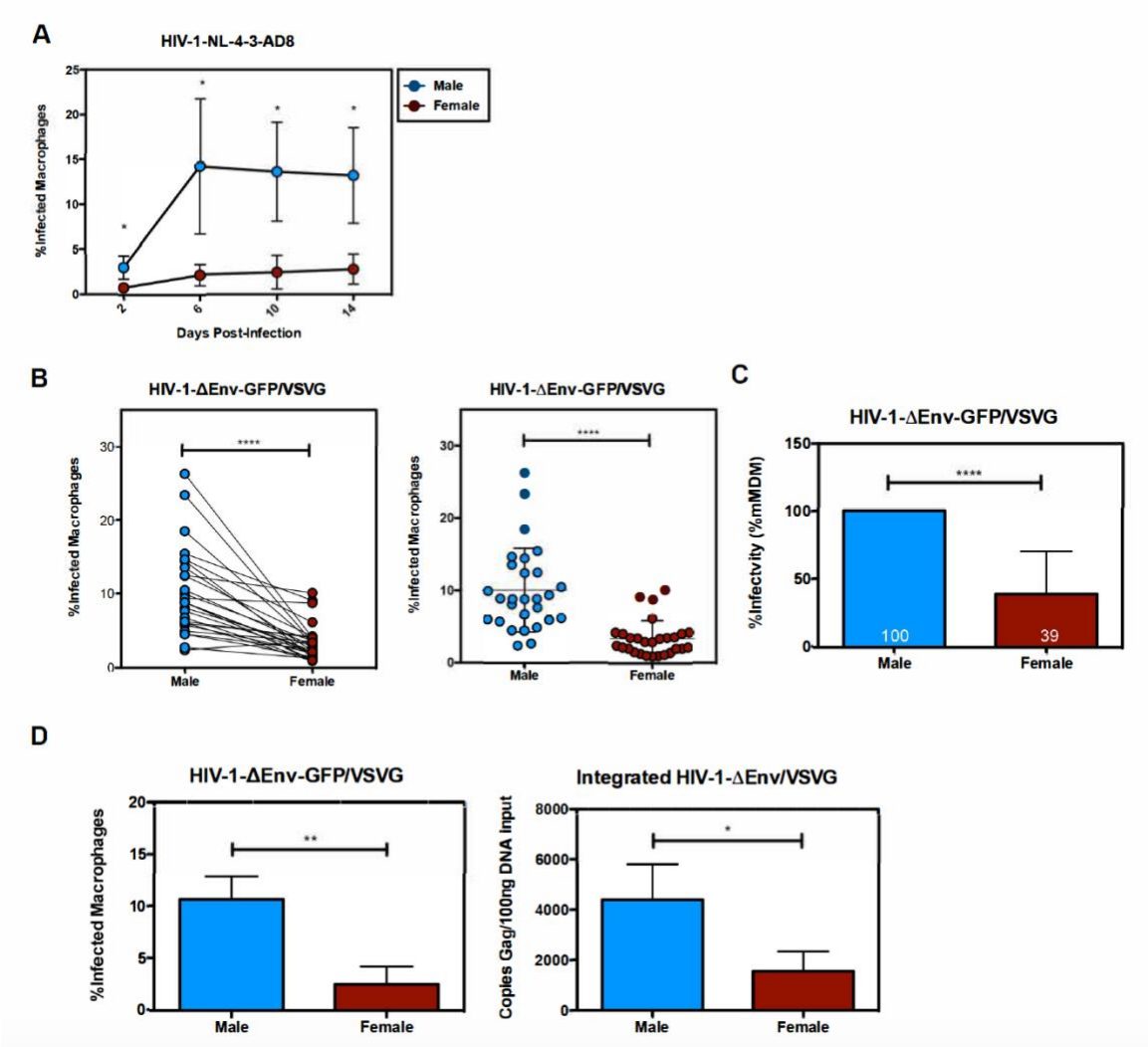

A
HIV-1-NL-4-3-BaL-IRES-HSA
%Infected Macrophages
**
30
20
10
0
Male
Female
B
HIV-1-NL-4-3-AD8
%Infected Macrophages
*
15
10
5
0
Male
Female
C
HIV-1-NL-4-3-BaL-IRES-HSA
%Infected Macrophages
****
150
100
50
0
100
25
Male
Female
D
HIV-1-NL-4-3-AD8
%Infectivity (% mMDM)
***
150
100
50
0
100
37
Male
Female

Figure 2.2. Sex differences in HIV-1 infection occur early and independent of virus entry. (A) Monocytes from six healthy donors (three male and three female) were isolated on a single draw date and differentiated simultaneously. Day 7 macrophages were infected with HIV-1-NL-4-3-AD8 and analyzed for intracellular p24 staining on days 2, 6, 10, and 14. Three male donors and three female donors were averaged to produce a single infection curve. (B) MDM were infected on day 7 with HIV-1−ΔEnv-GFP/VSVG and analyzed 48 hours later for GFP expression. Values represented as percentage of GFP+ cells as analyzed by flow cytometry. (C) Infections were analyzed pairwise and unpaired. Infection in male-derived MDM was set to 100% and percentage of infection in female-derived MDM was analyzed for each pair. (D) MDM from six healthy donors (three males and three females) were infected with HIV-1−ΔEnv-GFP/VSVG and analyzed 48 hours postinfection for GFP expression. Genomic DNA was isolated from MDM and analyzed for HIV-1 integration events via gag-PCR.


A
HIV-1-NL-4-3-AD8
%Infected Macrophages
Days Post-Infection
Male
Female
B
HIV-1-ΔEnv-GFP/VSVG
%Infected Macrophages
Male
Female
HIV-1-ΔEnv-GFP/VSVG
%Infected Macrophages
Male
Female
C
HIV-1-ΔEnv-GFP/VSVG
%Infectvity (%mMDM)
100
39
Male
Female
D
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Copies Gag/100ng DNA Input
Male
Female

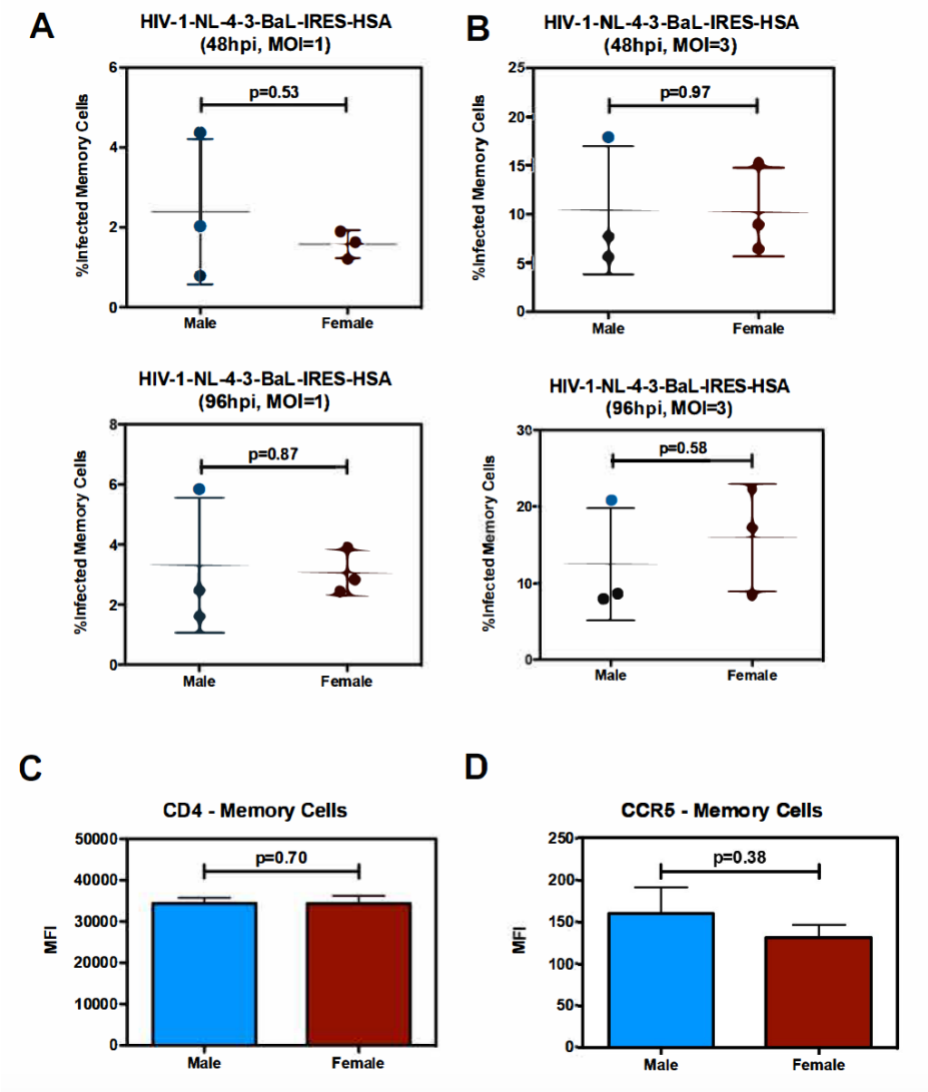

Figure 2.3. Memory CD4+ T cells do not exhibit sex-dependent differences in infectivity. (A) Memory CD4+ T cells were isolated from healthy donors and infected at an MOI=1 with HIV-1-NL-4-3-BaL-IRES-HSA and analyzed at 48 and 96 hours post-infection. (B) Memory CD4+ T cells isolated from healthy donors, infected at MOI=3, and analyzed at 48 and 96 hours postinfection. (C) Memory CD4+ cells were stained for cell surface expression of CD4 and CCR5 and analyzed via flow cytometry.

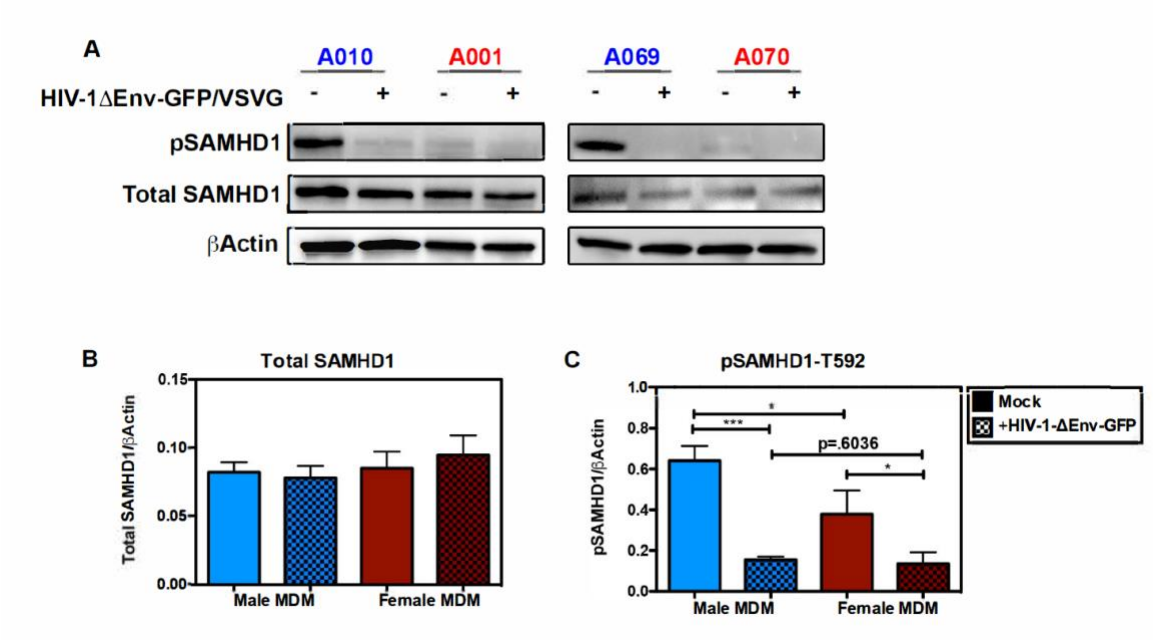

Figure 2.4. Sex differences in MDM infectivity occur prior to integration and correlate with differences in SAMHD1 activity. (A) Representative Western Blot images of two representative male/female-derived MDM pairs mock infected or infected with HIV-1−ΔEnv-GFP/VSVG probed for pSAMHD1-T592, Total SAMHD1, and β-Actin. (B) Western Blot quantification of six separate MDM (three males and three females) isolated and differentiated simultaneously analyzed for total SAMHD1. (C) Western Blot quantification of MDM from (B) analyzed for pSAMHD1.

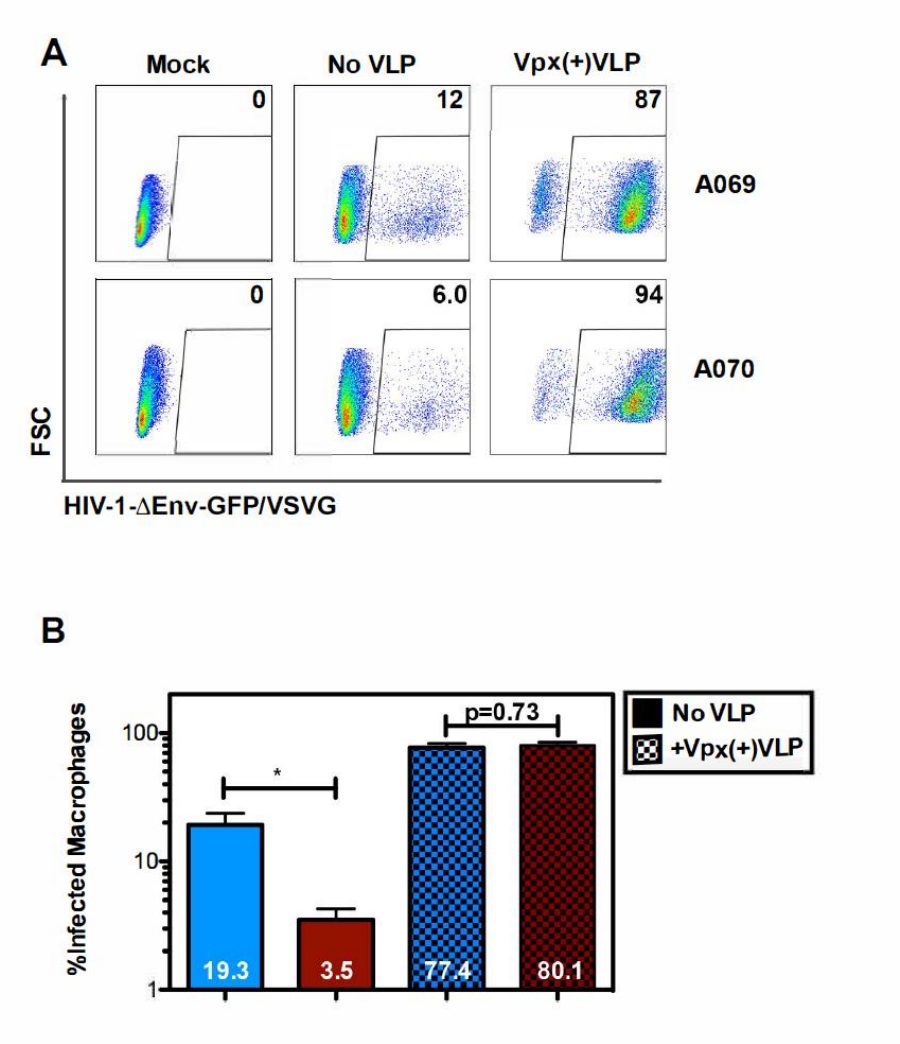

Figure 2.5. Sex differences in MDM infectivity are overcome via SAMHD1 bypass or Vpx-mediated SAMHD1 degradation. (A) Representative flow plots of MDM infected with HIV-1–ΔEnv-GFP/VSVG following pretreatment with Vpx(+)VLPs. (C) Quantification of six donors (three males and three females) infected with HIV-1–ΔEnv-GFP/VSVG in the presence or absence of Vpx(+)VLPs.

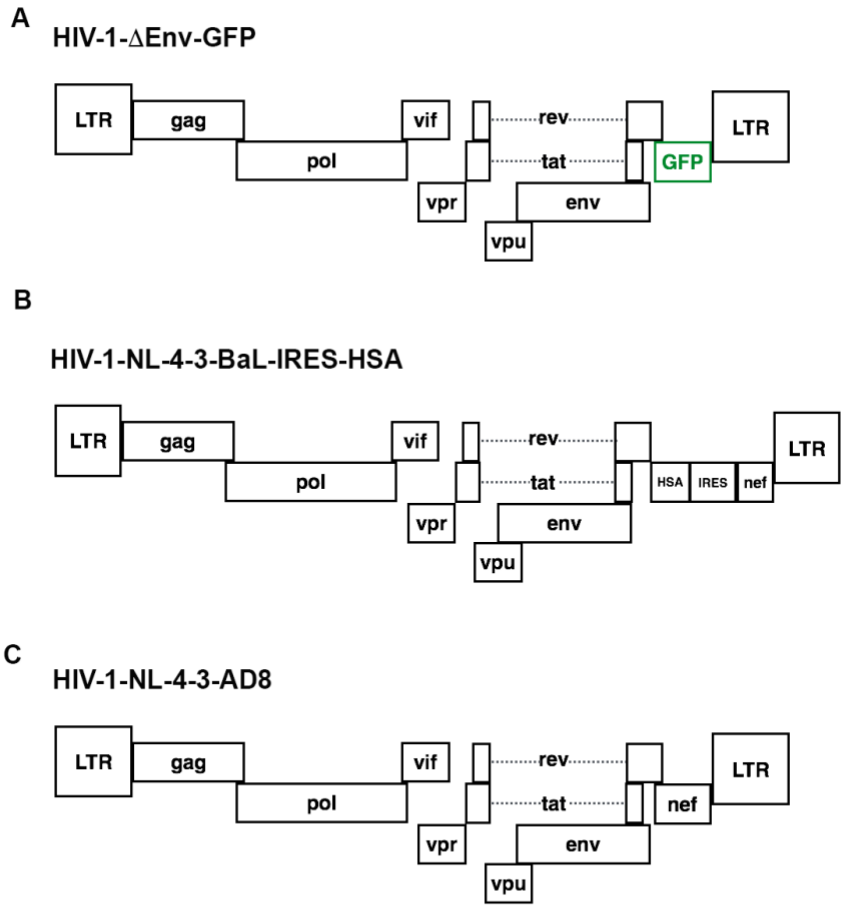

Figure S2.1. HIV-1 constructs. (A) HIV-1–ΔEnv-GFP/VSVG (B) HIV-1-NL-4-3-BAL-IRES-HSA (C) HIV-1-NL-4-3-AD8

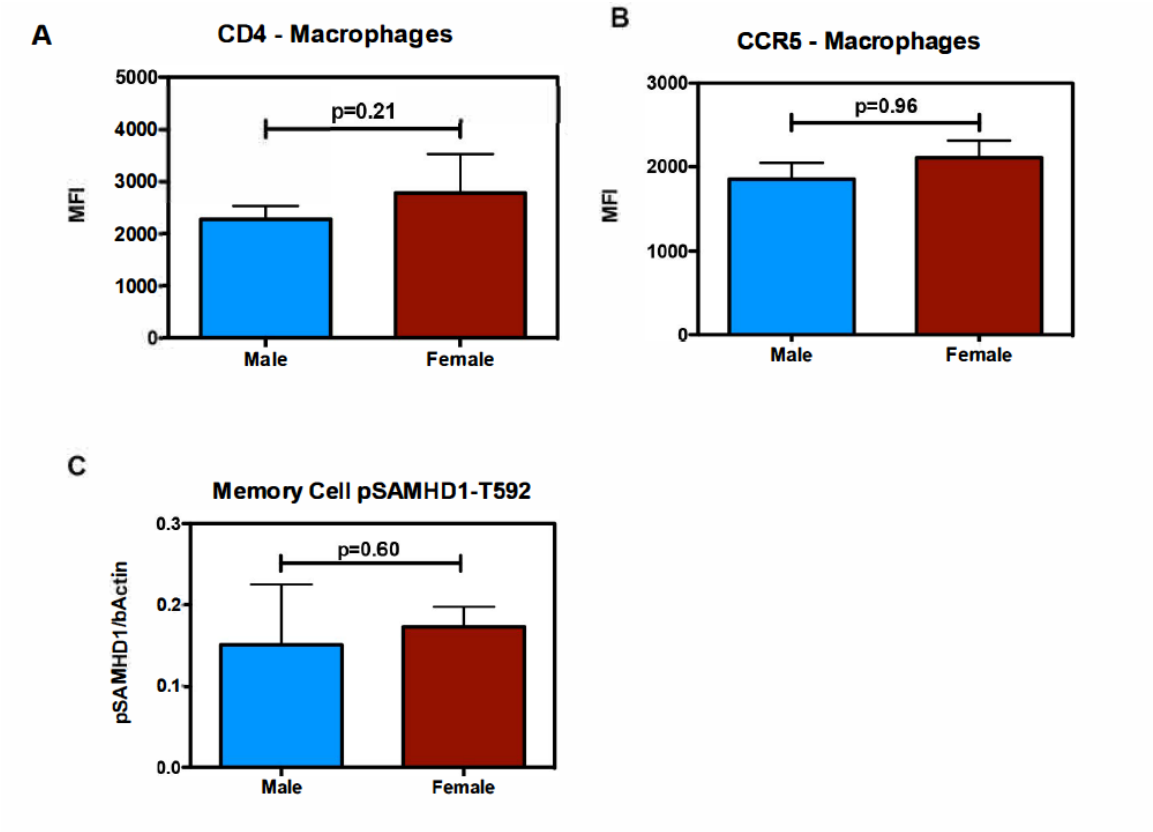

Figure S2.2. Entry does not play a role in sex-dependent differences in MDM infectivity or CD4+ T cell phosphorylation. (A) MDM in six healthy donor-derived macrophages from were analyzed for CD4 and CCR5 expression via flow cytometry. (B) Analysis of pSAMHD1 levels by Western Blot in six memory CD4+ T cells isolated from six donors (three males and three females).

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CHAPTER 3

SAMHD1 PHOSPHORYLATION COORDINATES THE ANTI-HIV-1 RESPONSE BY DIVERSE IFNS

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Abstract

Macrophages are susceptible to HIV-1 infection despite abundant expression of a variety of antiviral proteins, perhaps most importantly the restriction factor sterile alpha motif domain and histidine/aspartic-acid domain-containing protein 1 (SAMHD1). We investigated the role of SAMHD1 and its phosphodependent regulation in the context of HIV-1 infection in primary human monocyte-derived macrophages and the ability of various interferons and pharmacologic agents to modulate SAMHD1. Here we show that stimulation by type I, type II, and type III interferons converges upon activation of SAMHD1 via dephosphorylation at Threonine-592. CDK1, a known effector kinase for SAMHD1, was transcriptionally downregulated by all IFN types tested. Pharmacologic inhibition or siRNA-mediated knockdown of CDK1 phenocopied the effects of IFN on SAMHD1. A panel of FDA-approved tyrosine kinase inhibitors potently induced activation of SAMHD1 and subsequent HIV-1 inhibition. The viral restriction enforced via IFNs or Dasatinib could be overcome through bypass of SAMHD1, proving that their effects are exerted primarily through this pathway. Our results demonstrate that SAMHD1 activation, but not and increases in mRNA or protein induction, is the predominant mechanism of HIV-1 restriction induced by type I, type II and type III IFN signaling in macrophages, and presents a pharmacologically actionable target through which HIV-1 infection can be subverted.

Introduction

Macrophages are major targets of HIV-1 and their importance in infection establishment, progression, and persistence has been studied at length [1, 2]. They are

important contributors to virus amplification and dissemination during primary infection, and in the central nervous system (CNS), HIV-1-infected macrophages and microglia precipitate a spectrum of neurological impairments that persist in the setting of antiretroviral therapy[3-5]. It is known that macrophages exhibit low permissiveness to infection, in part due to the constitutive expression of the antiviral restriction factor SAM domain- and HD domain-containing protein 1 (SAMHD1), a deoxynucleoside triphosphate (dNTP) triphosphohydrolase which restricts HIV-1 by maintaining dNTP concentrations below the threshold required for efficient reverse transcription (RT)[6-8]. SAMHD1 is regulated at the level of phosphorylation by type I interferon (IFN), but to what extent type II and III IFNs affect SAMHD1 phosphorylation, and whether SAMHD1 is required for their activity, remains unknown [9].

IFNs play an important role in preventing viral infection through multiple effector mechanisms. Numerous studies have pointed to enhanced expression of various restriction factors that act at multiple stages of HIV-1 infection, including APOBEC-family proteins, MX2, GBP5, and others, to explain the potent antiviral effects of IFNs [10-13]. In this context, activation of interferon regulatory factor (IRF) family of transcription factors, nuclear translocation, and recognition of a conserved DNA sequence known as an IFN-stimulated response element (ISRE) present in the promoter-enhancer region of a number of genes direct a number of antiviral programs [14, 15]. Though IFNα and IFNβ are perhaps the most well-studied type I IFNs, numerous others exist in humans including IFNε, whose relevance in HIV-1 restriction has been demonstrated recently in macrophages [16]. The sole member of the type II IFN family, IFNγ, is widely recognized for its role in macrophage activation, and was recently shown

to induce a potent, Env-dependent block to HIV-1 infection in CD4+ T cells that was distinct from that induced by type I IFN [17]. The Type III IFNs, which include IFNλ1, λ2, and λ3, have been shown to exhibit anti-HIV-1 activity, and may be relevant in specific tissue sites such as the vaginal mucosa [18]. Macrophages are highly responsive to the modulating effects of many diverse interferons and interferon-like molecules. However, the mechanisms underlying the potent anti-HIV-1 capacities of diverse IFNs remain incompletely defined [19].

In this study, we examined the abilities of divergent IFN families to inhibit HIV-1 infection in primary human MDM, and provide mechanistic insight into the major effector function of type I, type II, and type III IFNs. We extended the study to explore the HIV-1 restriction potential of a panel of FDA-approved tyrosine kinase inhibitors (TKI), in an effort to define SAMHD1 as a potential pharmacologic target for anti-HIV-1 efforts. These studies show that SAMHD1 serves as the key regulator of HIV-1 infectivity in MDM whose activity is rapidly inducible at the level of protein dephosphorylation.

Results

Type I, type II, and type III IFNs induce SAMHD1-T592 dephosphorylation, resulting in enhanced restriction of HIV-1 infection in MDM

We sought to determine whether IFNs from various families could differentially modulate SAMHD1 protein levels or activity and whether these changes would in turn affect HIV-1 infection in macrophages. CD14+ monocytes were isolated from peripheral blood mononuclear cells (PBMC) from healthy donors and generated MDM following 7-

day differentiation. MDM were exposed for 24 hours to 50ng/mL concentrations of IFNα, IFNε, IFNγ, or IFNλ. Macrophages were infected with a replication-competent R5-tropic recombinant virus (HIV-1-BAL-HSA; Figure S2.1) [20] and analyzed for CD24 (HSA, mouse heat stable antigen) expression by flow cytometry 48 hours later (Figure 3.1). Without IFN treatment, infection levels ranged between 1 and 16% among donors. Because of the high degree of variability in infectivity among donors, we set infection levels in the absence of IFN to 100% for each individual donor, then normalized infection in the presence of IFN to that value (Figure 3.1). In each case, IFN treatment restricted infection, albeit to varying degrees: IFNα, IFNε, and IFNγ potently suppressed infectivity. In contrast, IFNλ reduced infectivity modestly and in a highly variable manner, a degree of protection that could not be enhanced by increasing concentrations of IFNλ (Supplemental Figure 3.2).

To determine whether the effects of IFN are dependent on viral entry, we utilized a replication defective virus pseudotyped with vesicular stomatitis virus glycoprotein (HIV-1ΔEnv-GFP/VSVG; Figure S2.1). VSVG binds to the low-density lipoprotein receptor for entry and shunts the virus towards the endocytic pathway, bypassing membrane fusion resulting from gp120 interaction with CCR5 [21]. HIV-1ΔEnv-GFP/VSVG was sensitive to each IFN tested, with IFNλ exhibiting the least potency and highest variability donor-to-donor, suggesting that the antiviral effector mechanisms induced by various IFNs are independent of both the route of viral entry and virus production (Figure 3.1).

To examine the role of SAMHD1 in restriction by the various IFN types, we generated cell lysates from MDM treated with IFN for 24 hours and probed for total

SAMHD1 and pSAMHD1-T592 (Figure 3.1; two representative donors shown). Addition of recombinant IFNα, IFNγ, or IFNλ led to a reduction in SAMHD1 phosphorylation that correlated directly with reduced infectivity by HIV-1ΔEnv-GFP/VSVG ($p<0.0001$, $R^2=0.9783$; Figure 3.1). The levels of total SAMHD1 protein were not affected by any of the IFNs tested, and there was no correlation between total SAMHD1 protein and infectivity following IFN stimulation (Figure 3.1). SAMHD1 activation through T592 dephosphorylation is thus a conserved effector mechanism resulting from stimulation of MDM by different IFN types.

SAMHD1 is active at baseline and controls MDM resistance to HIV-1

We hypothesized that highly permissive MDM would exhibit a higher degree of phosphorylation in SAMHD1, relative to the levels of total SAMHD1 protein. We derived MDM from six healthy donors and exposed them to HIV-1ΔEnv-GFP/VSVG. In the absence of stimulation, MDM from these donors exhibited vastly different levels of SAMHD1 phosphorylation (Figure 3.2) at baseline which correlated directly with the levels of infectivity ($p=0.045$, $R^2=0.6748$; Figure 3.2). Infectivity did not correlate with levels of total SAMHD1 protein (Figure 3.2). Although a modest correlation between CCR5 cell surface expression and infectivity was observed, differences in infectivity at baseline cannot be attributed to potential fluctuations in CD4 or CCR5 because the virus used in these experiments was pseudotyped with VSVG (Data Not Shown). The finding that MDM from healthy donors exhibit diverse signatures of SAMHD1 activity that correlate directly with infectivity supports our model in which infectivity in MDM depends on SAMHD1 activity and not expression.

SAMHD1 phosphorylation status is sufficient to explain IFN-induced HIV-1 restriction in primary MDM

To evaluate whether SAMHD1 is the relevant antiviral effector responsible for the HIV-1 restriction induced by IFNs, we delivered the SIVmac accessory protein Vpx to ectopically induce SAMHD1 degradation [6-8, 22-24]. We generated VSVG-pseudotyped virus-like particles (VLP) containing the SIVmac accessory protein Vpx (Vpx(+)VLP) and, as a control, VLPs lacking Vpx (Vpx(-)VLP) [8, 22].

Cells were exposed to Vpx(+)VLPs or Vpx(-)VLPs for 6 hours then treated for 18 hours with IFN. After VLP addition, cells were infected with HIV-1ΔEnv-GFP/VSVG. SAMHD1 degradation by Vpx(+)VLP and Vpx(-)VLP was evaluated by Western Blot at 24 hours post-VLP addition and infection levels were evaluated by flow cytometry 48 hours postinfection. Vpx(+)VLPs led to efficient SAMHD1 degradation, and addition of Vpx(+)VLPs effectively reversed the block to infection with HIV-1ΔEnv-GFP/VSVG while Vpx(-)VLPs failed to promote infection (Figure 3.3, one representative donor; additional donors shown in Figure 3.3). Vpx(+)VLP-mediated reversal of the IFN-induced antiviral state occurred in the context of SAMHD1-T592 dephosphorylation and without overt changes in SAMHD1 total protein levels (Figure 3.3). Cells not treated with IFN also demonstrated increased infectivity when treated with Vpx(+)VLP, consistent with previous reports showing that Vpx can overcome the restriction imposed by SAMHD1 at baseline, in the absence of exogenous IFN stimulation, proving that SAMHD1 is at least partially active under unstimulated conditions (Figure 3.3) [22].

SAMHD1 has two reported antiviral activities: it has been proposed to act both as a nucleotide triphosphohydrolase and as a ribonuclease [25-27]. To determine whether

SAMHD1 activation results in a state of HIV-1 restriction that relies on limiting dNTP, we exposed cells to high concentrations of deoxynucleosides (dNs) in the culture medium prior to infection. We predicted that exogenous dN administration would phenocopy the effect of Vpx(+)VLPs and reverse the IFN-induced antiviral activity. Deoxyribonucleosides (dNs) are cell-membrane permeable and are converted to dNTPs once inside the cell (Lahouassa, 2012, 22327569). Addition of dNs to the medium enhanced infection in the absence of IFN and completely relieved the IFN-induced restriction in IFN-treated cells (Figure 3.3; two representative donors shown). These results, together with those in Figures 3.3, establish SAMHD1 dephosphorylation as the principal effector mechanism against HIV-1 in the response of MDM to types I, II, and III IFN, which results in dNTP depletion and impaired HIV-1 RT.

Canonical HIV-1 restriction factors, but not SAMHD1, are transcriptionally induced by type I, type II, and type III IFN stimulation

We next sought to ascertain whether SAMHD1 was a bona fide type I, type II, or type III ISG in MDM, or whether SAMHD1-dependent, IFN-induced HIV-1 restriction could be attributed to post-translational protein modifications only. MDM were left untreated or stimulated with IFNα, IFNε, and IFNγ, or IFNλ. mRNA was isolated 18 hours following stimulation and subject to RNA-sequencing. Figure 3.4 shows the Log2 fold-change (sequence reads in IFN treatment over those without IFN) in mRNA for SAMHD1 along with a small subset of known ISGs (ISG15, ABOBEC3A, SIGLEC-1, and MX1, MX2, OAS1), cell cycle regulators known to act on SAMHD1 (CDK1, 2, 4 and 6) and immune-related genes (CD4, CCR5, CXCR1, CD180). SAMHD1 mRNA

levels were not dramatically affected by any of the IFN treatments (1.08-fold (IFNα), -1.24-fold (IFNε), 1.27-fold (IFNγ), and 1.21-fold (IFNλ)). In contrast, all tested ISGs were strongly induced by each of the IFNs tested, exhibiting expression levels that ranged between 5-fold (ISG15 by IFNγ) and 750-fold (APOBEC3A by IFNα) over the no-IFN condition (Figure 3.4).

Among the cyclin-dependent kinases examined in our RNAseq experiment, CDK1 mRNA was decreased 5.7-fold, 3.9-fold, and 20.8-fold after IFNα, IFNε, and IFNγ, respectively (Figure 3.4). CDK1 transcripts were modestly upregulated by IFNλ (1.4-fold), the only IFN with modest to undetectable HIV-1 restriction in our studies. Therefore, we speculated that downregulation of CDK1 by IFNα, IFNε, and IFNγ is part of the mechanism by which these IFNs induce dephosphorylation of SAMHD1. To verify this prediction, we tested the CDK1/CDK2 BMS-265246 inhibitor for its ability to restrict HIV-1ΔEnv-GFP/VSVG infection. BMS-265246 restricted HIV-1 infection in MDM, whereas GW2580, a TKI that targets CSF-1R among other RTKs, had no effect (Figure 3.5) [28].

We next performed siRNA to CDK1 to verify its role in SAMHD1 phosphorylation and HIV-1 restriction. Knockdown of CDK1 resulted in a significant reduction in SAMHD1 phosphorylation, concomitant with a reduction in MDM susceptibility to HIV-1, while siRNA targeting SAMHD1 led to an enhanced susceptibility of MDM to HIV-1 (Figures 3.5). In agreement with our findings with Vpx(+)VLPs, knockdown of SAMHD1 similarly reversed the IFN-induced restriction to HIV-1, suggesting the majority of the antiviral effect is exerted through SAMHD1 activation (Figure 3.5).

SAMHD1 activity can be regulated pharmacologically

Because activation of SAMHD1 imposes a potent blockade against HIV-1, it would be ideal if the activity of SAMHD1 could be controlled pharmacologically. Recently, it was shown that this can be accomplished in CD4+ T cells with the use of Dasatinib, a Bcr-Abl-specific TKI FDA-approved for use in chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ AML) [29, 30]. We tested a panel of tyrosine kinase inhibitors to determine whether they possess anti-HIV-1 activity and whether that activity stems from SAMHD1 dephosphorylation. Among the TKIs tested were Dasatinib (DAS), Bosutinib (BOS), Crenolanib (CRE), Palbociclib (PAL), and Ponatinib (PON). These compounds are FDA-approved and target a variety of cellular pathways including Abl Kinase, Src-family kinases, type III receptor tyrosine kinases, as well as CDK4 and CDK6 [31]. Each inhibitor led to HIV-1 restriction, with DAS providing the most potent HIV-1 blockade (Figure 3.6). HIV-1ΔEnv-GFP/VSVG infectivity was inversely correlated with the proportion of SAMHD1 present in its activated, dephosphorylated state as manipulated by the TKIs (Figure 3.6). We focused on Dasatinib to test whether SAMHD1 phosphorylation and infectivity exhibited a dose-dependent relationship, and observed overt changes in pSAMHD1-T592 that corresponded to increasing concentrations (Figure 3.6). The dephosphorylation of SAMHD1 was concomitant with and inversely proportional to the infectivity by HIV-1ΔEnv-GFP/VSVG (Figure 3.6).

TKIs, including DAS, target diverse cellular pathways at high nanomolar concentrations [32]. If SAMHD1 is indeed responsible for the effects of the TKIs tested, then incubation with Vpx(+)VLP following treatment should induce degradation of

SAMHD1 and overcome the TKI-imposed restriction. Incubation with DAS rendered cells highly resistant to infection (0.5%) when compared with untreated cells (6.0%), representing a 92% protection (Figure 3.6). Addition of Vpx(+)VLP to untreated cells enhanced infection 8.8-fold (6.0% to 53.0%) while addition of Vpx(+)VLP in the context of DAS enhanced infection 76-fold (0.5% to 38%), representing a reversal of the DAS-induced antiviral state. Taken together, our experiments establish a causal link between SAMHD1 activation and protection from HIV-1 infectivity in MDM, and provide evidence to support a dynamic regulatory role for SAMHD1 in MDM. Furthermore, we demonstrate that SAMHD1 activity can be manipulated pharmacologically to render macrophages refractory to HIV-1 infection.

Materials and methods

Isolation of healthy donor PBMCs

Healthy donors age 18 and older were recruited for this study under the University of Utah Institutional Review Board (IRB) protocol #676637. Written informed consent was obtained from all donors. Whole blood was obtained by peripheral phlebotomy and peripheral blood mononuclear cells (PBMC) were isolated using a Lymphoprep density gradient (Stemcell Technologies).

Generation and infection of MDM

CD14+ monocytes were isolated via positive selection with magnetic beads (Miltenyi Biotec). Cells were allowed to adhere in serum-free media for 2 hours, which was then removed and replaced with RPMI + 10% pooled human serum (Innovative

Research). Media was changed at day 5, and cells were cultured for a total of 7 days to allow differentiation to MDM prior to experimentation as previously described. MDM were infected with 250ng of either HIV-1-BAL-HSA or HIV-1ΔEnv-GFP/VSVG as determined by p24 ELISA for 6 hours. Cells were washed twice with fresh media to remove unbound virus. Infection was quantified via flow cytometry at 48 hours post-infection.

Generation of viruses

Replication-defective virus (HIV-1ΔEnv-GFP/VSVG) was generated using calcium-phosphate-mediated transfection of HEK293T cells. Briefly, HIV-1ΔEnv-GFP and VSVG plasmids were co-transfected for 6 hours. Transfection media was removed and cells were cultured over 2 days, with virus-containing supernatants removed at 24 and 48 hours post-transfection. These viruses contain a frameshift mutation in envelope and are capable of only a single-round infection when Env is provided in trans as previously described [33]. Replication-competent virus (HIV-1-BAL-HSA) was generated through a similar transfection protocol using a single plasmid (pNL-43-BAL-IRES-HSA) courtesy of Dr. Michel Tremblay (Centre Hospitalier de l'Université Laval). All viruses were quantified using p24 ELISA (Zeptometrix) and stored at -80° Celsius until further use.

RNAseq analysis for ISGs

MDM were generated as described. and stimulated with 25ng/mL of the indicated IFNs. Total RNA was isolated 18 hours following stimulation using RNeasy

Mini Kit (Qiagen). Intact poly(A) RNA was purified from total RNA samples (100-500 ng) with oligo(dT) magnetic beads and stranded mRNA sequencing libraries were prepared as described using the Illumina TruSeq Stranded mRNA Library Preparation Kit (RS-122-2101, RS-122-2102). Purified libraries were qualified on an Agilent Technologies 2200 TapeStation using a D1000 ScreenTape assay (cat# 5067-5582 and 5067-5583). The molarity of adapter-modified molecules was defined by quantitative PCR using the Kapa Biosystems Kapa Library Quant Kit (cat#KK4824). Individual libraries were normalized to 10 nM and equal volumes were pooled in preparation for Illumina sequence analysis. Sequencing libraries (25 pM) were chemically denatured and applied to an Illumina HiSeq v4 single read flow cell using an Illumina cBot. Hybridized molecules were clonally amplified and annealed to sequencing primers with reagents from an Illumina HiSeq SR Cluster Kit v4-cBot (GD-401-4001). Following transfer of the flowcell to an Illumina HiSeq 2500 instrument (HCSv2.2.38 and RTA v1.18.61), a 50 cycle single-read sequence run was performed using HiSeq SBS Kit v4 sequencing reagents (FC-401-4002).

Inhibitors and IFNs

Recombinant human IFNα (PBL Assay Science), IFNε (EnQuire Bio), IFNγ (Peprotech), and IFNλ (Peprotech) were purchased from the indicated suppliers, resuspended at 50ng/μL, and added to cell culture at the indicated concentrations. Inhibitors were purchased from suppliers and resuspended at a concentration of 1mM and added to culture at a concentration of 1μM.

Discussion

Our experiments demonstrate that SAMHD1 dephosphorylation at threonine-592 represents a central mechanism of HIV-1 restriction that is common to the three known families of IFNs, though with vastly different efficiencies, as evidenced by the limited potency of IFNλ. Enhanced dNTPase activity appears to be the functional outcome of SAMHD1 activation, supported by several lines of evidence, including complete reversal of the IFN-induced restriction by addition of exogenous dNs and degradation of SAMHD1 by the SIV protein Vpx [8, 22]. The finding that diverse IFNs are functional to the degree to which they modulate SAMHD1 phosphorylation and activation without effecting changes in total protein is an important step in understanding innate immune responses in macrophages. We further report a previously undescribed activity of the type I IFNε which display antiviral activity comparable to other type I and type II IFNs, as well as the type III IFNλ, that activate SAMHD1. While the ability of IFNε to induce a state of HIV-1 resistance in macrophages involving generation of reactive oxygen species has been recently reported, we identify its major effector function in SAMHD1 activation [16]. Finally, we show that HIV-1 inhibition by various FDA-approved TKIs is dependent on the activation of SAMHD1, providing important mechanistic understanding as to how these compounds act and can be directed towards HIV-1 cure efforts.

Macrophages are important targets of HIV-1 infection in vivo, and their significance to infection establishment and viral persistence is only beginning to be understood. It has been speculated that macrophages can support HIV-1 infection and harbor virus over prolonged periods of time independent of T cells, even in the setting of ART, a hypothesis that has recently been strengthened by experiments conducted in

humanized myeloid-only mice [1, 34]. Therefore, strategies that aim to prevent virus spread to tissue macrophages, either independently or in the context of latency reversal strategies, will be important components of ongoing HIV-1 cure efforts.

SAMHD1 was first identified as the human homolog of a previously described mouse IFNγ-inducible GTP-binding protein known as MG11[35, 36]. Recently, SAMHD1 has been shown to be induced by stimulation with type I and type II IFNs via downregulation of miR-181 and miR-30a in human monocytes [37]. A similar phenotype was observed in astrocytes and microglia and was also dependent on miR181a [38]. In hepatocytes, it has been shown that IFN can induce SAMHD1 transcription, inducing an antiviral state that restricts Hepatitis B Virus (HBV) infection [39]. Additionally, it has been shown that in mature DCs, co-culture with lymphocytes can lead to downregulation of SAMHD1 and enhance DC permissiveness to HIV-1 [40]. In the present study, we show that the type I, II, and II IFN-induced HIV-1 restriction in MDM is not derived from changes in SAMHD1 protein or mRNA levels, and that the antiviral state hinges upon changes in SAMHD1 activity as determined by T592 phosphorylation.

It has been previously reported that IFNα is a potent modulator of HIV-1 infectivity that acts at a preintegration step [41] dependent on the ubiquitin-proteasome pathway [42]. These observations came prior to the discovery that SAMHD1 was the cellular protein counteracted by Vpx [7, 8]. It was later shown that in dendritic cells, the IFNα-induced block to HIV-1 involved, at least in part, SAMHD1[43]. In cycling cells, SAMHD1 can be activated through T592 dephosphorylation in the presence of IFNα [9]. Most recently, it has been appreciated that artificial ‘stimulation’ of MDM through the addition of fetal bovine serum during differentiation can push MDM toward a G1-like

state that renders them more permissive to HIV-1, concomitant with an increase in SAMHD1 T592 phosphorylation and expression of other proteins responsible for cell-cycle regulation [44]. While the signals responsible for the G1-like state in MDM remained uncharacterized, it is clear that MDM respond to mitogenic stimuli despite their status as terminally differentiated, postmitotic cells.

The activity of SAMHD1 in lymphocytes is regulated by cyclin/cyclin-dependent kinase-mediated (CDK) threonine-592 phosphorylation in a cell cycle-dependent manner [9]. However, SAMHD1 phosphorylation can also be regulated independent of cell division [44]. MDM differentiate from monocytes to assume a postmitotic state. Therefore, it is striking that cell-cycle regulatory elements that could be viewed as dispensable in these cells are not only present but maintain a critical role in regulating SAMHD1 phosphorylation in response to IFN stimulation and also in response to DNA damage [44]. The specific CDKs responsible for SAMHD1 kinase activity depend on cell type, with CDK1, CDK2, CDK4, and CDK6 all demonstrating a regulatory effect on SAMHD1 in different contexts [9, 45, 46]. Phosphorylation of residue T592 impaired SAMHD1 tetramerization resulting in diminished capacity for dNTP hydrolysis and impaired anti-HIV-1 activity [47, 48]. Our results reveal that IFN-induced activation of SAMHD1 is effected via downregulation of CDK1 mRNA and suggest that in the absence of stimulation, CDK1 maintains SAMHD1 phosphorylated and, to a large extent, inactive. While it is formally possible that other IFN-unresponsive cell-cycle-related proteins act on SAMHD1, further studies will be required to determine their specific contribution to SAMHD1 dynamics in macrophages [45, 46].

The finding that diverse IFNs converge on SAMHD1 activation reveals a

common denominator in IFN signaling, despite differences in expression of numerous other documented antiviral genes in response to IFN stimulation [10-13]. Importantly, our study shows that SAMHD1 activation serves as a critical function of diverse IFN signaling that drives their antiviral capacity. While we did not directly address the input of other IFN-stimulated antiviral genes, such as APOBEC-family proteins or MX2, these genes are not targeted by SIVmac/smm Vpx. Indeed, a recent study showed that SIV of red-capped mangabeys and mandrills (SIVrcm/mnd-2) enhance HIV-1 infection in resting CD4+ T cells in a SAMHD1- and dNTP-pool independent manner. However, Vpx from these strains were unable to enhance infection in macrophages and also failed to induce degradation or dephosphorylation of SAMHD1 [49]. Thus, the near-complete abrogation of IFN antiviral activity by SIVmac Vpx implicates SAMHD1 as the major player in the IFN-indued antiviral state.

SAMHD1 has recently been proposed as a potential tumor suppressor, and in fact, many FDA-approved anticancer TKIs have potent effects on SAMHD1 activation in vitro and in vivo [29, 30, 46]. In cancer, SAMHD1 is thought to play a role in controlling cell cycle of tumor cells, where its activity as a triphosphohydrolase can restrain uncontrolled cellular proliferation by blunting cellular DNA synthesis [50]. Expanding our study beyond the natural biology of HIV-1 infection, we show that SAMHD1 activity can be targeted by several FDA-approved anticancer TKIs. TKIs exerted their anti-HIV-1 activity through SAMHD1-T592 dephosphorylation, revealing two possibilities regarding their activity. Though not formally tested, it will be interesting to understand whether the anti-HIV-1 activity of TKIs results from direct inhibition of CDKs known to phosphorylate SAMHD1, or whether kinase cascades affected by TKIs converge on

CDKs to maintain steady-state levels of SAMHD1 phosphorylation and inactivity. The results of our inhibitor studies, using TKIs known to target receptor tyrosine kinases (RTK), would suggest that signaling events originating at the cell surface may be crucial determinants of SAMHD1 dynamics in macrophages. While the precise signals leading to changes in SAMHD1 activity remain unknown, future studies aimed at delineating these pathways will be important in understanding how SAMHD1 activity is controlled at steady state and can be manipulated therapeutically. Targeting the relevant pathways may prove useful in anti-HIV-1 efforts or in preventing end-organ damage observed in patients on ART, including HIV-1 associated neurologic dysfunction.

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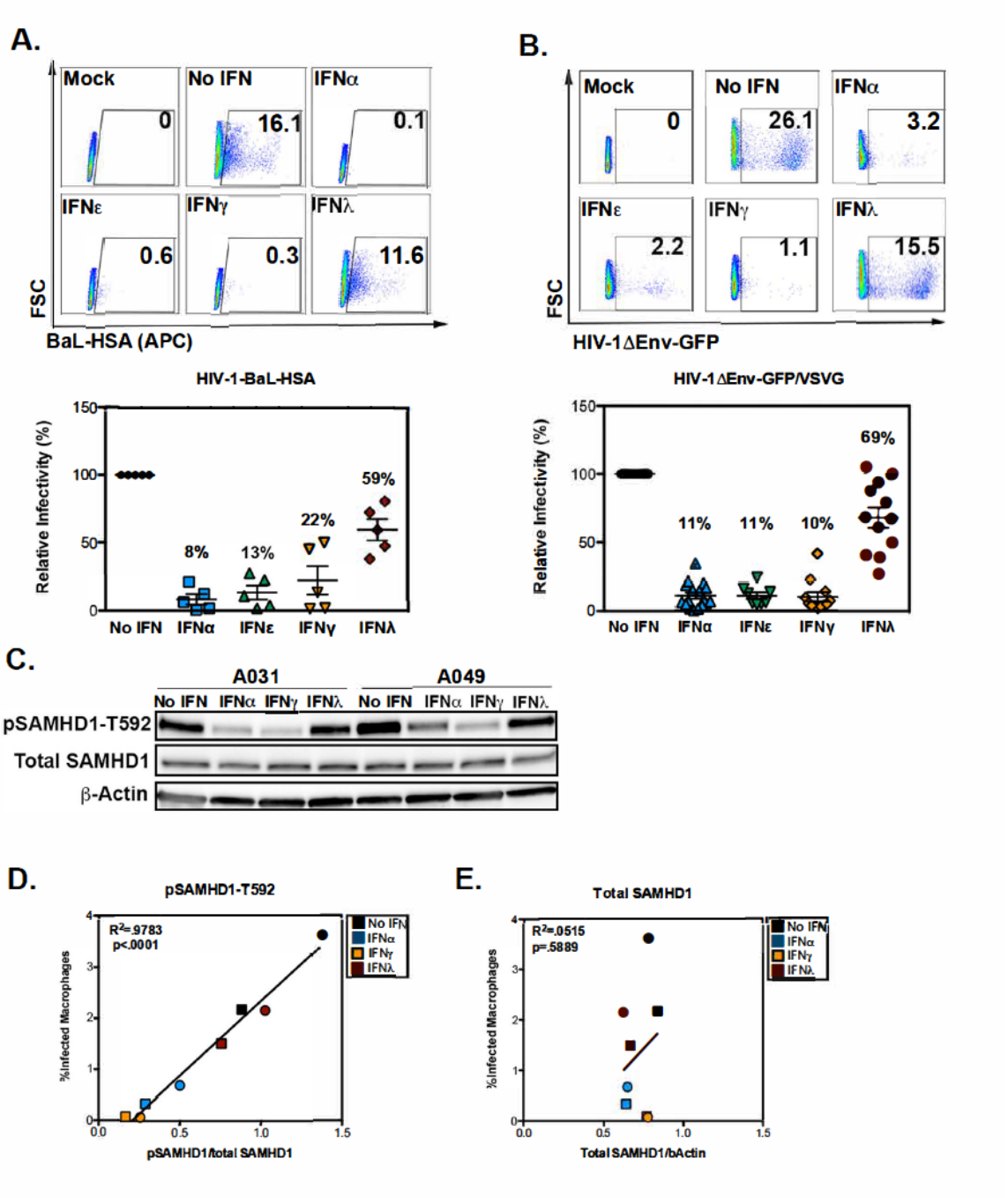
Author contributions

Conceptualization, M.A.S. and V.P.; Methodology, M.A.S. and V.P., and J.E.C.; Investigation, M.A.S and V.P.; Writing – Original Draft, M.A.S. and V.P.; Writing – Review & Editing, M.A.S, V.P., A.M.S., T.H., and A.B.; Funding Acquisition, M.A.S. and V.P.; Resources, M.T.; Supervision, V.P., A.M.S., and A.B.

Figure 3.1. Diverse IFNs restrict HIV-1 in MDM and induce SAMHD1 dephosphorylation. (A) Flow cytometric analysis of MDM infected with replication-competent HIV-1-BAL-HSA. Numbers indicate %CD24+ cells. (B) Flow cytometric analysis of MDM infected with HIV-1ΔEnv-GFP/VSVG. Numbers indicate %GFP+ cells. (C) MDM lysates (10μg) from representative donors (A031 and A049) were analyzed by Western Blot for pSAMHD1-T592, total SAMHD1, and β-actin. (D) Correlation between pSAMHD1-T592 and %GFP+ or (E) total SAMHD1 and %GFP+ MDM for A031 (circles) and A049 (squares).


A.
Mock
No IFN
IFNα
IFNε
IFNγ
IFNλ
0
16.1
0.1
0.6
0.3
11.6
FSC
BaL-HSA (APC)
B.
Mock
No IFN
IFNα
IFNε
IFNγ
IFNλ
0
26.1
3.2
2.2
1.1
15.5
FSC
HIV-1ΔEnv-GFP
HIV-1-BaL-HSA
Relative Infectivity (%)
150
100
50
0
8%
13%
22%
59%
No IFN
IFNα
IFNε
IFNγ
IFNλ
HIV-1ΔEnv-GFP/VSVG
Relative Infectivity (%)
150
100
50
0
11%
11%
10%
69%
No IFN
IFNα
IFNε
IFNγ
IFNλ
C.
A031
A049
No IFN
IFNα
IFNγ
IFNλ
No IFN
IFNα
IFNγ
IFNλ
pSAMHD1-T592
Total SAMHD1
β-Actin
D.
pSAMHD1-T592
R²=.9783
p<.0001
No IFN
IFNα
IFNγ
IFNλ
%Infected Macrophages
pSAMHD1/total SAMHD1
E.
Total SAMHD1
R²=.0515
p=.5889
No IFN
IFNα
IFNγ
IFNλ
%Infected Macrophages
Total SAMHD1/bActin

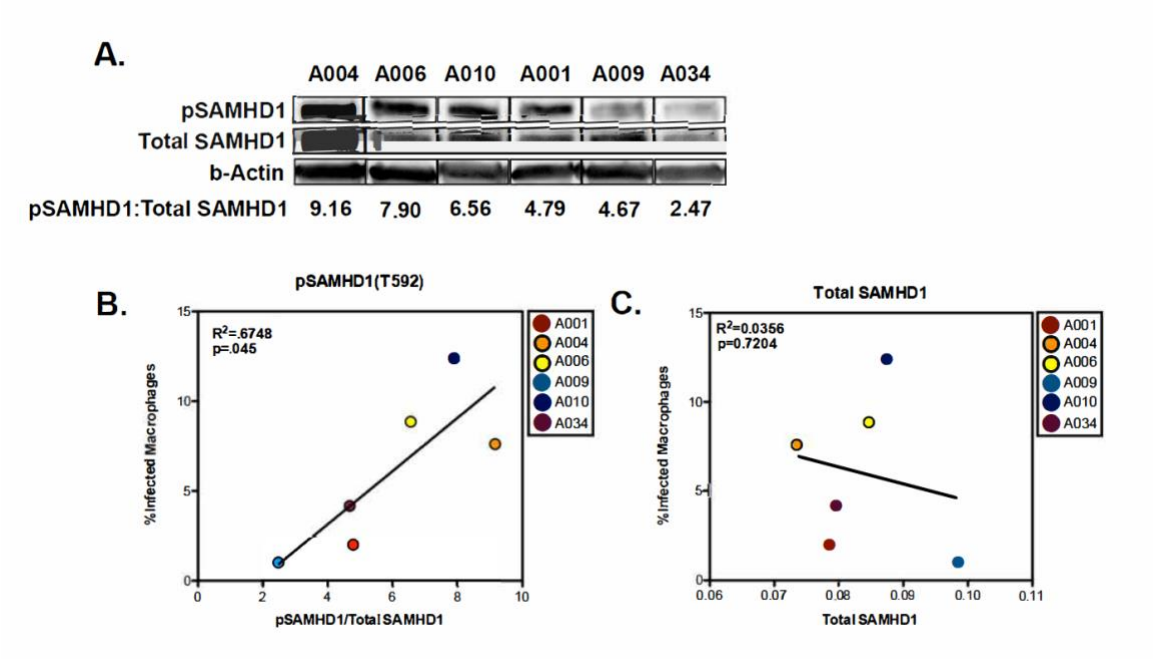
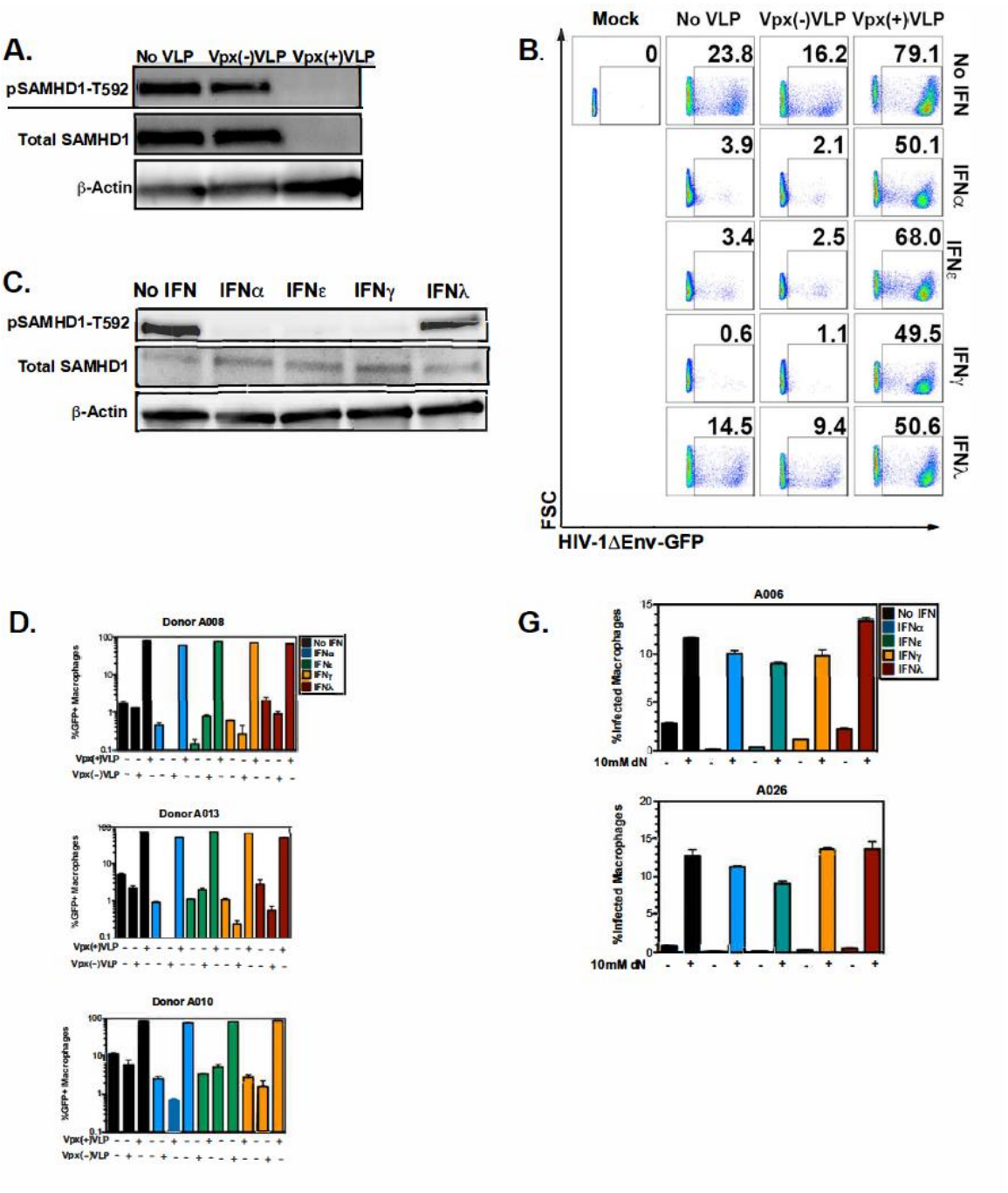


Figure 3.2. Macrophages exhibit diverse pSAMHD1-T592 signatures at baseline that correlate with infectivity. (A) MDM lysates from n=6 donors were analyzed by Western Blot in for pSAMHD1-T592, total SAMHD1, and actin. Numbers indicate the proportion of pSAMHD1-T592/total SAMHD1 as calculated by Western Blot densitometry. Lanes were cut following development of a single membrane to remove alternating lanes. (B) Correlation between pSAMHD1-T592 and %GFP+ macrophages as analyzed by flow cytometry. (C) Correlation between total SAMHD1 and %GFP+ MDM.

Figure 3.3. SAMHD1 is the major downstream effector of diverse IFN types. (A) Representative Western Blot analysis of No VLP-, Vpx(-)VLP-, and Vpx(+)VLP-treated MDM extracts probed for pSAMHD1-T592, Total SAMHD1, and β-actin. (B) Representative flow cytometric analysis of viable MDM infected with a replication-incompetent HIV-1ΔEnv-GFP/VSVG in the presence or absence of 50ng/mL IFNα, IFNε, IFNγ, or IFNλ, (rows) and treated with the Vpx VLP indicated (columns). Numbers indicate percentage of events falling within the GFP+ gate as established with mock infected MDM. (C) Western Blot analysis of pSAMHD1-T592, total SAMHD1, and actin for donor A051. (D) Bar graphs of n=3 additional, independent donors with each condition conducted in triplicate. (E) Data summary showing Fraction Infected with each IFN treatment in the absence of Vpx VLPs. (F) Data summary showing Fraction Infected with each IFN treatment in the presence of Vpx VLPs. (G). Donors A006 and A026 treated with IFN and exposed to 10mM dN then infected with HIV-1ΔEnv-GFP/VSVG. Bars indicate the %GFP-positive cells within the viable population as measured by flow cytometry.


A.
No VLP
Vpx(-)VLP
Vpx(+)VLP
pSAMHD1-T592
Total SAMHD1
β-Actin
B.
Mock
No VLP
Vpx(-)VLP
Vpx(+)VLP
0
23.8
16.2
79.1
No IFN
3.9
2.1
50.1
IFNα
3.4
2.5
68.0
IFNε
0.6
1.1
49.5
IFNγ
14.5
9.4
50.6
IFNλ
FSC
HIV-1ΔEnv-GFP
C.
No IFN
IFNα
IFNε
IFNγ
IFNλ
pSAMHD1-T592
Total SAMHD1
β-Actin
D.
Donor A008
Donor A013
Donor A010
%GFP+ Macrophages
Vpx(+)VLP
Vpx(-)VLP
G.
A006
A026
%Infected Macrophages
10mM dN

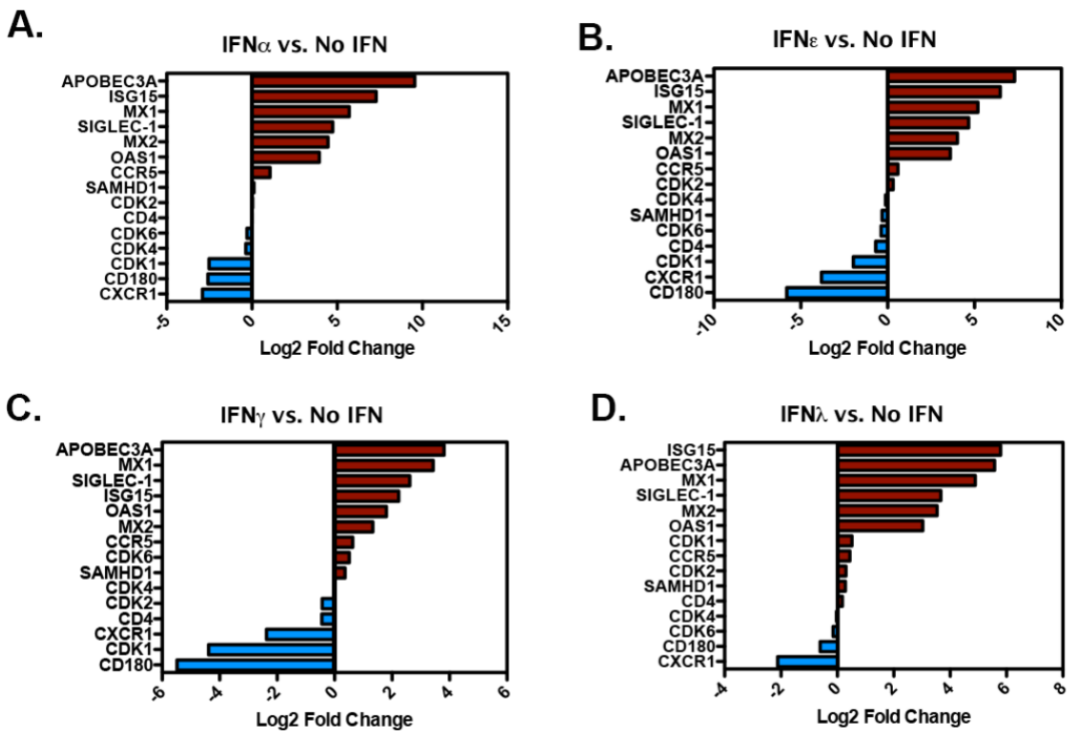
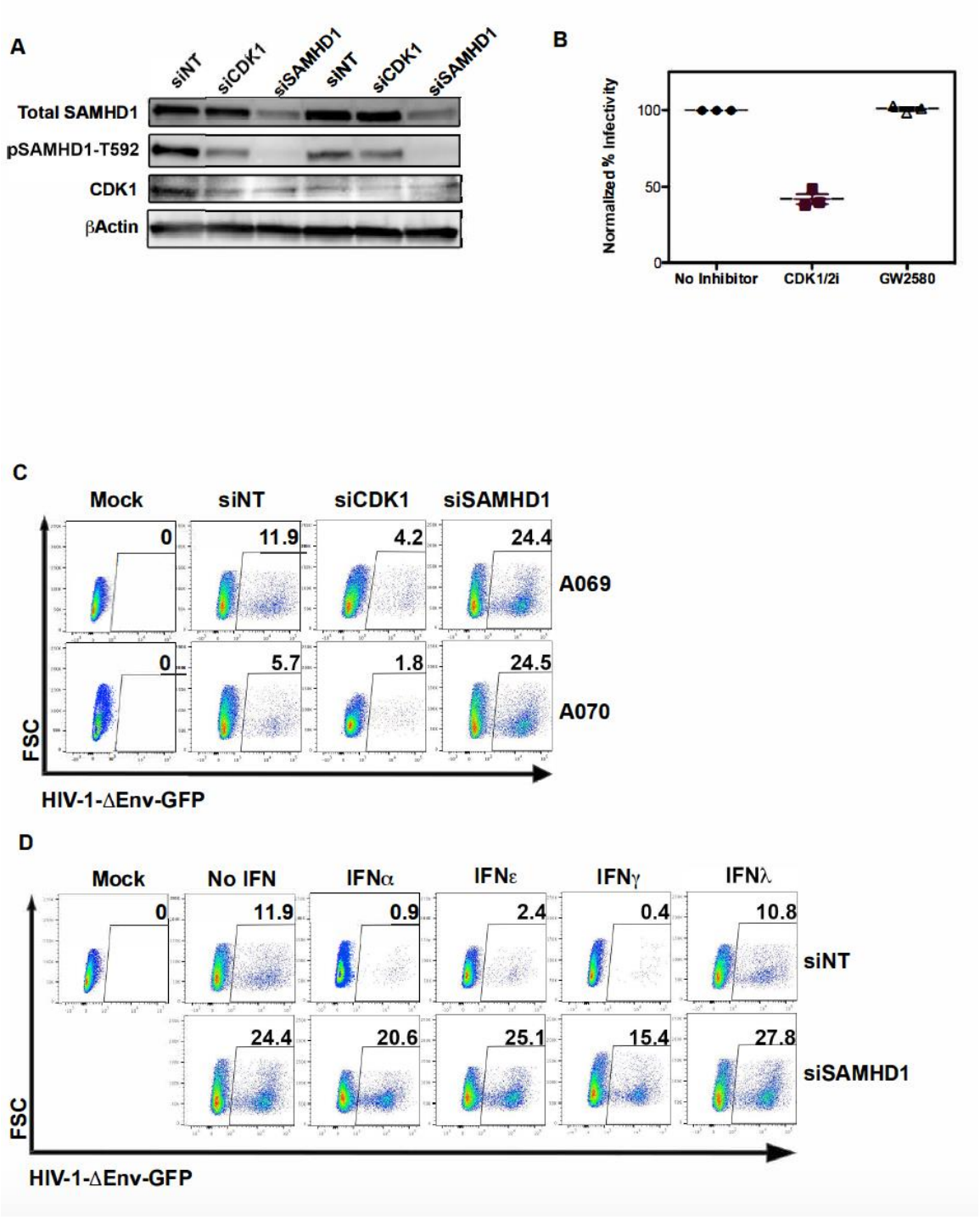


Figure 3.4. SAMHD1 is not a canonical ISG in MDM. (A-D). Gene expression signatures of select genes represented as Log2 Fold Change relative to the untreated condition (no IFN) for IFNα, IFNε, IFNγ, and IFNλ.

Figure 3.5. siRNA to CDK1 and SAMHD1 reveal central roles in IFN-induced HIV-1 restriction. (A) Western Blot analysis of siRNA-treated MDM (densitometry bar graphs (B) The effect of CDK1/2 inhibition on infection with HIV-1ΔEnv-GFP/VSVG was determined by flow cytometry, with infection in untreated cells set to 100%. N=3 representative donors shown. (C) Representative siRNA flow cytometry plots from two donors. (D) Representative flow cytometry plots from IFN-treated MDM with and without SAMHD1 knockdown.


A
siNT
siCDK1
siSAMHD1
siNT
siCDK1
siSAMHD1
Total SAMHD1
pSAMHD1-T592
CDK1
βActin
B
Normalized % infectivity
100
50
0
No Inhibitor
CDK1/2i
GW2580
C
Mock
siNT
siCDK1
siSAMHD1
0
11.9
4.2
24.4
A069
0
5.7
1.8
24.5
A070
FSC
HIV-1-ΔEnv-GFP
D
Mock
No IFN
IFNα
IFNε
IFNγ
IFNλ
0
11.9
0.9
2.4
0.4
10.8
siNT
24.4
20.6
25.1
15.4
27.8
siSAMHD1
FSC
HIV-1-ΔEnv-GFP

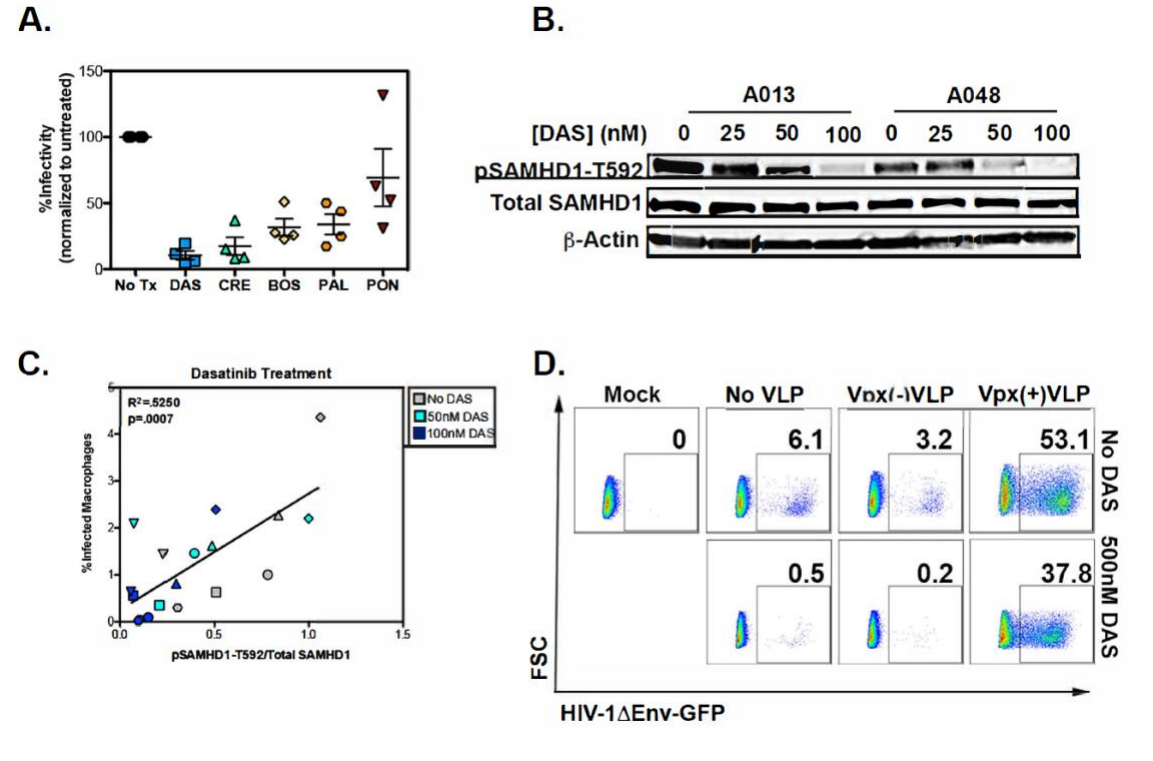


Figure 3.6. SAMHD1 is a required for TKI-induced restriction. (A) Summary flow cytometric analysis of HIV-1 infection in four separate donors treated with various TKIs. Values indicate the %GFP-positive cells within the viable population of cells infected with HIV-1ΔEnv-GFP/VSVG. (B) Western Blot analysis of MDM lysates from two representative donors treated with the indicated concentration of Dasatinib. (C) Correlation between relative pSAMHD1-T592 and infectivity for six donors treated with 0nM (gray), 50nM (light blue), and 100nM (dark blue) Dasatinib. (D) MDM treated with 500nM Dasatinib and treated with the Vpx VLP indicated. Numbers represent the percentage of live cells within the GFP+ gate as established with mock infected MDM.

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CHAPTER 4

DECIPHERING MECHANISMS OF HIV-1 SENSING IN MACROPHAGES

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Abstract

Macrophages are major targets of HIV-1 and contribute substantially to the anti-viral response following infection. As innate immune cells, macrophages sense and respond to viral infection through a variety of mechanisms that often converge on the production and release of interferons. Interferons trigger multiple host defenses aimed at achieving an antiviral state, including expression of numerous interferon-stimulated genes that target HIV-1 at multiple stages of the virus life cycle. A major consequence of interferon signaling is the activation of SAMHD1. Here, we show that like type I and type II interferons, infection with HIV-1 elicits SAMHD1 activation and induces an anti-viral state that protects bystander macrophages from subsequent challenge. Interestingly, infection with HIV-1 fails to induce significant levels of interferons at the transcriptional level. In this study, we aim to delineate the precise mechanisms by which HIV-1 is sensed in primary macrophages and the signaling pathway leading to SAMHD1 dephosphorylation. Furthermore, we wish to establish a mechanistic basis for SAMHD1 activation in the presence of interferons, infection with HIV-1, or tyrosine kinase inhibition which phenocopies these effects.

Introduction

A major consequence of HIV-1 recognition by immune cells is the production of proinflammatory cytokines, which results in a state of chronic inflammation in both treated and untreated HIV-1 infection [1, 2]. Macrophages are equipped with numerous mechanisms responsible for sensing viral infection and inducing responses that prevent viral spread and propagation [3]. They serve as major sources of inflammatory cytokine

production during infection, including IL-6 and TNF-α, which are important components of the host antiviral defense, but can be associated with increased risk to conditions such as neurologic, cardiovascular, liver, bone, and kidney disease [2, 4]. While the cytokines and cell types responsible for the double-edged sword of persistent inflammation and inhibition of ongoing virus replication during HIV-1 infection are well described, the precise signals that lead to these responses are the subject of ongoing investigation. Furthermore, whether HIV-1 is capable of active suppression of immune recognition and subsequent evasion of the antiviral response remains an open question.

An important group of sensors involved in recognition of diverse viruses are the Toll-Like Receptors (TLRs). Embedded within the plasma membrane or that of endosomes, TLRs are members in a vast family of sensors known collectively as pattern recognition receptors (PRRs), due to their ability to detect common moieties present on the surface of infectious microbes [5, 6]. Due to their proximity to the cell surface and endosomal compartments, TLRs are among the first lines of defense against microbial infection. TLR3, TLR7, and TLR8 recognize single-stranded (ss) and double-stranded (ds) RNA, and are thought to contribute to HIV-1 sensing in cells of the myeloid lineage, specifically plasmacytoid dendritic cells (pDC) [7, 8]. TLR9 recognizes viral DNA, and is theoretically capable of sensing HIV-1 early during infection. However, because the majority of reverse transcription (RT) and generation of HIV-1 DNA occurs in the cytoplasm of host cells, TLR9 is unlikely to contribute meaningfully to the immune response to HIV-1 infection [9]. In the majority of infection settings, TLRs that sense foreign nucleic acids are capable of inducing IFN responses through activation of interferon regulatory factor (IRF)-family transcription factors [10]. Indeed, pDC-

mediated sensing of HIV-1 via TLR7 during acute infection is thought to be a major driver of the IFN response observed [11]. During progressive infection, levels of circulating pDCs are greatly diminished, and it is thought that other cell types, such as macrophages, are responsible for persistent inflammation. However, failure of HIV-1 to induce potent proinflammatory responses in macrophages and myeloid-lineage cells raises key questions in the ability of HIV-1 to circumvent these defenses.

Following entry into the host cell, viral components, otherwise referred to as pathogen associated molecular patterns (PAMPs), become exposed to a variety of sensors. Chief among these sensors in myeloid cells is cyclic GMP-AMP synthase (cGAS), which activates type I IFN production after binding viral DNA through synthesis of cGAMP and subsequent activation of stimulator of interferon genes (STING). STING activation triggers phosphorylation of interferon regulator factor 3 (IRF3) through activation of the serine/threonine kinase TANK-binding kinase 1 (TBK1). Recent studies have uncovered a role for cGAS-STING-mediated HIV-1 recognition in the setting of the HIV-2 accessory protein Vpx. Vpx targets SAMHD1 for ubiquitin-mediated degradation during infection with HIV-2, and is thus provided in trans during in vitro infection with HIV-1 to render macrophages highly susceptible to infection. However, a role for this signaling axis in ‘natural’ infection with HIV-1 in the absence of Vpx, and whether superinfection is required for sensing, remains to be determined.

In this report, we begin to delineate the mechanisms of HIV-1 recognition in primary human monocyte-derived macrophages through two major approaches: phospho-proteomic analysis of HIV-1-infected MDM and transcriptomic analysis by RNA-sequencing (RNAseq) of HIV-1-stimulated genes. We report that infection with HIV-1

induces activation of SAMHD1 and elicits a state of protection from subsequent challenge despite the absence of a detectable IFN response. Expanding on our previous findings outlining a central role for SAMHD1 in regulating HIV-1 infection, we further sought to characterize the mechanism of SAMHD1 activation and subsequent protection against HIV-1 in the presence of various stimuli: HIV-1 challenge, type I, II, and III IFN treatments, and tyrosine kinase inhibition by Dasatinib. Here, we begin to unravel several pathways that provide insight into HIV-1 sensing by macrophages and the salient mechanisms responsible for IFN- and Dasatinib-induced MDM resistance to infection. In future studies, we aim to address the dependence of these antiviral responses on SAMHD1 activation.

Results

MDM infection with HIV-1 elicits SAMHD1 activation and protection from subsequent viral challenge

We first sought to determine whether infection with HIV-1 would elicit a state of protection from subsequent virus challenge. To this end, we generated healthy donor MDM and infected with either HIV-1ΔEnv-GFP/VSVG (replication-incompetent) or HIV-1-BaL-HSA (replication-competent). Subsequent challenge with HIV-1ΔEnv-mCherry/VSVG 48 hours later revealed a significant protective effect, such that initial infection with HIV-1 exhibited inhibition of subsequent HIV-1 infection (Figure 4.1). Interestingly, this protection was not limited to cells that had been previously infected, as determined by mCherry reporter gene expression. This effect was observed whether virus contained native envelope or was VSVG-pseudotyped, indicating that route of entry is

not a major factor in determining protection from subsequent challenge (Figure 4.1). The protective effect did not appear to require virus replication, as cells infected with envelope-deleted virus were protected at least as well as cells infected with virus containing native envelope.

We generated whole cell extracts for Western Blot analysis from cells infected with HIV-1ΔEnv-GFP/VSVG 48 hours postinfection, the same time point at which cells had been infected with subsequent HIV-1ΔEnv-mCherry/VSVG. Analysis of SAMHD1 activation revealed a marked dephosphorylation of SAMHD1 in the absence of changes in total SAMHD1 protein (Figure 4.1). On average, the levels of SAMHD1 phosphorylation decreased 87% following infection (0.45 mean normalized Western Blot densitometry for Mock-infected cells; 0.06 mean normalized Western Blot densitometry for HIV-1ΔEnv-GFP/VSVG-infected cells) (Figure 4.1). Analysis of secondary infection over multiple donors challenged initially with HIV-1ΔEnv-GFP/VSVG- or HIV-1-BAL-HSA showed significant restriction (21% residual infectivity, HIV-1ΔEnv-GFP/VSVG; 17% residual infectivity, HIV-1-BAL-HSA)(Figure 4.1). These results suggest that macrophages sense HIV-1 infection in MDM and mount a response that leads to SAMHD1 activation via dephosphorylation.

Experiments aimed at elucidating whether SAMHD1 activation is exclusive to infected cells, or whether soluble signaling molecules drive bystander activation, are ongoing. Utilizing HIV-1-BaL-HSA, which expresses surface-bound CD24 (HSA), we are currently carrying out cell-sorting experiments to distinguish infected cells (CD24+) from bystander cells (CD24-). Preliminary experiments wherein infected-cell supernatants were transferred to uninfected cell cultures suggests that cell contact may be

a requirement for cell-to-cell communication that leads to bystander cell protection, as supernatants alone did not appear to induce the degree of protection observed in previous tests (Figure 4.2). Interestingly, it has recently been appreciated that cGAMP(2'-5'), the second messenger essential for cGAS-STING-mediated IFN production, is passed to bystander cells via gap junctions through a mechanism depedent on cell-cell contact [12]. Preliminary studies in our lab have confirmed the effect of cGAMP(2'-3') on HIV-1 inhibition in MDM, though studies are ongoing to determine whether these are the relevant pathways involved in MDM sensing of HIV-1 and subsequent bystander protection (Figure 4.3).

Phospho-proteomic analysis of HIV-1-infected MDM reveals several key kinase pathways

We turned to phospho-proteomics analysis to identify signaling pathways that may be important for the recognition of HIV-1 and subsequent SAMHD1 activation. To this end, MDM were infected with HIV-1ΔEnv-GFP/VSVG. At 48 hours, infection was analyzed by reporter gene expression (GFP) and cells were lysed for Western Blot analysis. Additional cell extracts were sent for phosphoproteomic analysis utilizing the Kinexus Antibody Microarray KAM-900P platform. KAM-900P antibody microarray utilizes 613 phosphosite-specific antibodies and 265 pan-specific antibodies, allowing determination of changes in both total protein levels and regulatory residues that are manipulated by phosphorylation.

The results of the comparison between mock-infected and HIV-1ΔEnv-GFP/VSVG-infected cells are summarized (Table 4.1). Interestingly, the major cyclin-

dependent kinase (CDK) responsible for SAMHD1 phosphorylation, CDK1 was significantly altered [13]. CDK1 is itself subject to phospho-regulation at residues Y14 and Y15, which are known to negatively regulate CDK1 activity[13]. Phosphorylation of these specific regulatory residues was enhanced 6.58-fold following infection with HIV-1ΔEnv-GFP/VSVG, suggesting CDK1 inhibition may be a major contributor to SAMHD1 activation observed (Table 4.1, %CFC=558). A number of other pathways were indicated by the microarray, including growth factor signaling (EGFR, PDGFR). However inhibitor studies targeting these receptors failed to confirm a role for signaling in MDM (Data Not Shown). STRING protein interaction analysis revealed a central role for CDKs which are known to manipulate SAMHD1 activity, including CDK1, CDK2, and CDK4 (Figure 4.4). With these data, in concert with insights from our previous report, future investigations will focus on the role of CDK1 in SAMHD1 phosphorylation in order to better understand how a state of protection of MDM is established.

Comparative analysis of IFN-, HIV-1-, and Dasatinib-stimulated genes by RNAseq reveals differences and similarities in the antiviral response

We sought to identify changes in the transcriptional landscape in an effort to better understand the similarities and differences between IFN-, HIV-1-, and Dasatinib-mediated SAMHD1 activation and protection from infection with viral challenge. To this end, we generated MDM from the same donor used in the Kinexus Antibody Microarray experiment and isolated total mRNA following 24-hour treatment with the following: IFNα, IFNε, IFNγ, IFNλ, infection with HIV-1-BAL-HSA, or 500nM Dasatinib. Cell extracts were generated for Western Blot analysis in tandem and analyzed for SAMHD1

phosphorylation, which revealed activation (dephosphorylation) and protection from infection in accordance with previous reports (Donor A010, Figure 4.1). Analysis of CD24 (HSA) expression from HIV-1-BAL-HSA-infected cells revealed infection levels reaching 30%, which provided sufficient resolution for detection of transcriptional changes in a mixed population of infected (30%) and uninfected (70%) cells.

RNAseq revealed 83 genes found in all contrasts, and rlog differences were clustered (Figure 4.5). The values in the heatmap represent the amount a gene deviates in a specific sample from the gene's average across all samples. Hierarchical clustering showed similarities between type I and type II IFNs, which differed significantly from Dasatinib, type III IFN, and untreated cells. Unexpectedly, HIV-1-BAL-HSA-infected cells did not cluster with type I and type II IFNs, strongly suggesting that HIV-1 infection in MDM fails to induce an interferon-stimulated gene program. Further analysis revealed that only one IFN, IFNε, was modestly induced by HIV-1 infection (Data Not Shown). We selected a cutoff of Log2FC = +/-2.0 (a 4-fold induction of transcripts in either direction), and generated Venn Diagrams to better understand the similarities and differences in between HIV-1 infection and treatment with type I, II, and II IFNs. Strikingly, only 23 gene signatures were positively enriched in all treatments, and there were no shared genes exhibiting negative enrichment across all treatments (Figure 4.6). Pathway analysis of each treatment confirmed similarities between type I and type II IFNs, including positive enrichment for genes involved in interferonopathies such as Systemic Lupus Erythematosus and negative enrichment for genes involved in cell cycle control (Figure 4.6). Further analysis will be required to determine the key regulators of SAMHD1 control and protection afforded by initial infection with HIV-1, as well as

whether these regulators exhibit significant interdependency.

Materials and methods

Isolation of healthy donor PBMCs

Healthy donors age 18 and older were recruited for this study under the University of Utah Institutional Review Board (IRB) protocol #676637. Written informed consent was obtained from all donors. Whole blood was obtained by peripheral phlebotomy and peripheral blood mononuclear cells (PBMC) were isolated using a Lymphoprep density gradient (Stemcell Technologies).

Generation and infection of MDM

CD14+ monocytes were isolated via positive selection with magnetic beads (Miltenyi Biotec). Cells were allowed to adhere in serum-free media for 2 hours, which was then removed and replaced with RPMI + 10% pooled human serum (Innovative Research). Media was changed at day 5, and cells were cultured for a total of 7 days to allow differentiation to MDM prior to experimentation as previously described. MDM were infected with 250ng of either HIV-1-BAL-HSA or HIV-1ΔEnv-GFP/VSVG as determined by p24 ELISA for 6 hours. Cells were washed twice with fresh media to remove unbound virus. Infection was quantified via flow cytometry at 48 hours post-infection.

Generation of viruses

Replication-defective virus (HIV-1ΔEnv-GFP/VSVG) was generated using calcium-phosphate-mediated transfection of HEK293T cells. Briefly, HIV-1ΔEnv-GFP and VSVG plasmids were co-transfected for 6 hours. Transfection media was removed and cells were cultured over 2 days, with virus-containing supernatants removed at 24 and 48 hours posttransfection. These viruses contain a frameshift mutation in envelope and are capable of only a single-round infection when Env is provided in trans as previously described [14]. Replication-competent virus (HIV-1-BAL-HSA) was generated through a similar transfection protocol using a single plasmid (pNL-43-BAL-IRES-HSA) courtesy of Dr. Michel Tremblay (Centre Hospitalier de l'Université Laval). All viruses were quantified using p24 ELISA (Zeptometrix) and stored at -80° Celsius until further use.

RNAseq analysis for ISGs

MDM were generated as described and stimulated with 25ng/mL of the indicated IFNs. Total RNA was isolated 18 hours following stimulation using RNeasy Mini Kit (Qiagen). Intact poly(A) RNA was purified from total RNA samples (100-500 ng) with oligo(dT) magnetic beads and stranded mRNA sequencing libraries were prepared as described using the Illumina TruSeq Stranded mRNA Library Preparation Kit (RS-122-2101, RS-122-2102). Purified libraries were qualified on an Agilent Technologies 2200 TapeStation using a D1000 ScreenTape assay (cat# 5067-5582 and 5067-5583). The molarity of adapter-modified molecules was defined by quantitative PCR using the Kapa Biosystems Kapa Library Quant Kit (cat#KK4824). Individual libraries were normalized

to 10 nM and equal volumes were pooled in preparation for Illumina sequence analysis. Sequencing libraries (25 pM) were chemically denatured and applied to an Illumina HiSeq v4 single read flow cell using an Illumina cBot. Hybridized molecules were clonally amplified and annealed to sequencing primers with reagents from an Illumina HiSeq SR Cluster Kit v4-cBot (GD-401-4001). Following transfer of the flowcell to an Illumina HiSeq 2500 instrument (HCSv2.2.38 and RTA v1.18.61), a 50 cycle single-read sequence run was performed using HiSeq SBS Kit v4 sequencing reagents (FC-401-4002).

Discussion and perspectives

The field of HIV-1 biology has largely focused on the contribution of CD4+ T cells to host-pathogen interactions and, rightfully, on their importance to viral persistence and latency [14, 15]. While myeloid cells have long been recognized as an important target cell of HIV-1, the knowledge base related to infection of macrophages and dendritic cells remains comparatively sparse [16]. Much of this lack of insight is due to valid questions surrounding the in vivo role of macrophages [16]. Despite their ubiquitous presence in tissue sites important for HIV-1/AIDS pathogenesis, it is still unknown whether macrophages are capable of supporting a true state of latency, and whether they constitute a bona fide viral reservoir in vivo [17]. However, their role as motivators of pathogenic inflammatory responses in HIV-1/AIDS and as major targets of virus at transmission at mucosal sites warrants a deeper understanding of the complex relationship between myeloid cells and HIV-1 [2].

The ability of cells to sense HIV-1 constitutes a major component of innate immune responses, and is postulated to affect virus seeding at mucosal sites of transmission [18]. The inability of macrophages to respond to virus challenge by mounting a robust IFN response is an important area of future investigation, one that may provide a more complete picture of the mechanisms of transmission and point toward strategies to combat HIV-1. Understanding how macrophages respond to systemic HIV-1 infection by inducing proinflammatory responses, and whether those responses protect immune cells from subsequent infection, will be important in identifying pathways that may be targeted therapeutically to curb pathologies associated with ongoing virus replication and persistent inflammation.

Whether SAMHD1 activation is required for macrophage protection from subsequent challenge is an outstanding question. We will address this question through two major approaches: first, by identification of the viral PAMP required to trigger SAMHD1 activation, and second, through elimination of SAMHD1 prior to secondary infection. Preliminary experiments involving VSVG-pseudotyped HIV-1 particles suggest that the crucial recognition events for SAMHD1 activation occur independent of virus entry (Figure 4.1). Thus, efforts should be focused on nucleic acid components through inhibition of reverse transcription (to study RNA) or integration (to study DNA) to examine which, if any, nucleic acids are involved. By preventing recognition of the viral PAMP required for SAMHD1 dephosphorylation following infection, either through siRNA-mediated knockdown of sensors specific for recognition of RNA (RIG-I) or DNA (cGAS/STING), we hypothesize that subsequent infection with HIV-1 will be de-

restricted. A similar phenotype is expected through elimination of SAMHD1 using Vpx(+)VLPs or siRNA targeting SAMHD1.

It is clear that HIV-1 induces a response in MDM that resembles IFN-induced ISG stimulation. However, the induction of ISGs, particularly those with anti-HIV-1 activity, is largely blunted. A major question in the field remains regarding the mechanisms by which HIV-1 subverts innate immune responses. Indeed, a number of HIV-1 accessory proteins have been implicated in contributing to circumventing innate immune sensing. While these accessory proteins undoubtedly contribute to the blunted innate response to HIV-1, there are likely multiple mechanisms involved [19]. An intriguing possibility is the induction of noncoding RNAs by HIV-1, specifically those that act to dampen the IFN response to infection. We have shown that HIV-1 itself can induce the expression of a long noncoding RNA (lncRNA) in MDM, lncRNA-CMPK2. lncRNA-CMPK2, or Negative Regulator of Interferon Responses (NRIR), has been previously reported to be enriched in chronic hepatitis C virus (HCV) infected individuals [20]. This lncRNA also represses expression of ISGs in the context of type I IFN stimulation, and may be an additional mechanism by which HIV-1 counteracts the innate immune response [20]. Experiments aimed at elucidating the role of HIV-1-induced NRIR, including shRNA-mediated knockdown, are ongoing.

The experiments included in this dissertation demonstrate that SAMHD1 dephosphorylation at threonine-592 represents a central mechanism of HIV-1 restriction common to diverse families of IFNs. The finding that diverse IFNs are functional to the degree to which they modulate SAMHD1 phosphorylation and activation without effecting changes in total protein is an important step in understanding innate immune

responses in macrophages. We further show that use of FDA-approved TKIs leads to HIV-1 inhibition, and that this is dependent on the activation of SAMHD1, providing important mechanistic understanding as to how these compounds act and can be directed towards HIV-1 cure efforts. Whether sex-dependent differences are a result of differential baseline expression of IFN will be the subject of future investigation, though precedent has been set for sex-based differences in innate immune responses in other cell types[8].

The activity of SAMHD1 is regulated in a cell cycle-dependent manner, but can also be regulated independent of cell division, as reported in this dissertation [21]. It is striking that cell-cycle regulatory elements that could be viewed as dispensable in terminally-differentiated MDM are both present and critical in regulating SAMHD1 phosphorylation in response to IFN stimulation and HIV-1 infection. Our results reveal that IFN-induced activation of SAMHD1 is effected via downregulation of CDK1 mRNA and suggest that in the absence of stimulation, CDK1 maintains SAMHD1 phosphorylated and, to a large extent, inactive.

SAMHD1 is thought to play a role in controlling cell cycle of tumor cells, where its activity as a triphosphohydrolase can restrain uncontrolled cellular proliferation by blunting cellular DNA synthesis [22]. We show that SAMHD1 activity can be targeted by several FDA-approved anticancer TKIs. TKIs exerted their anti-HIV-1 activity through SAMHD1-T592 dephosphorylation, revealing two possibilities regarding their activity. It will be important to understand whether the anti-HIV-1 activity of TKIs results from direct inhibition of CDKs known to phosphorylate SAMHD1, or whether kinase cascades affected by TKIs converge on CDKs to maintain steady-state levels of SAMHD1

phosphorylation and inactivity. The results of our inhibitor studies, using TKIs known to target receptor tyrosine kinases (RTK), would suggest that signaling events originating at the cell surface may be crucial determinants of SAMHD1 dynamics in macrophages. Targeting the relevant pathways may prove useful in anti-HIV-1 efforts or in preventing end-organ damage observed in patients on ART, including HIV-1 associated neurologic dysfunction. An understanding of the fundamental mechanisms may further be translated to the field of oncology [22].

Macrophages are important targets of HIV-1 infection in vivo, and their significance to infection establishment and viral persistence is only beginning to be understood. It has been speculated that macrophages can support HIV-1 infection and harbor virus over prolonged periods of time independent of T cells, even in the setting of ART, a hypothesis that has recently been strengthened by experiments conducted in humanized myeloid-only mice. Therefore, strategies that aim to prevent virus spread to tissue macrophages, either independently or in the context of latency reversal strategies, will be important components of ongoing HIV-1 cure efforts. Understanding the fundamental pathways important for HIV-1 restriction, HIV-1 sensing, and innate immune responses will be crucial to the success of these ongoing efforts. Understanding the cellular and molecular determinants of sex-based differences will be crucial components of understanding the observed sex-dependent differences of HIV-1 infection in vivo and essential to therapeutic strategies aimed at curbing HIV-1 pathogenesis.

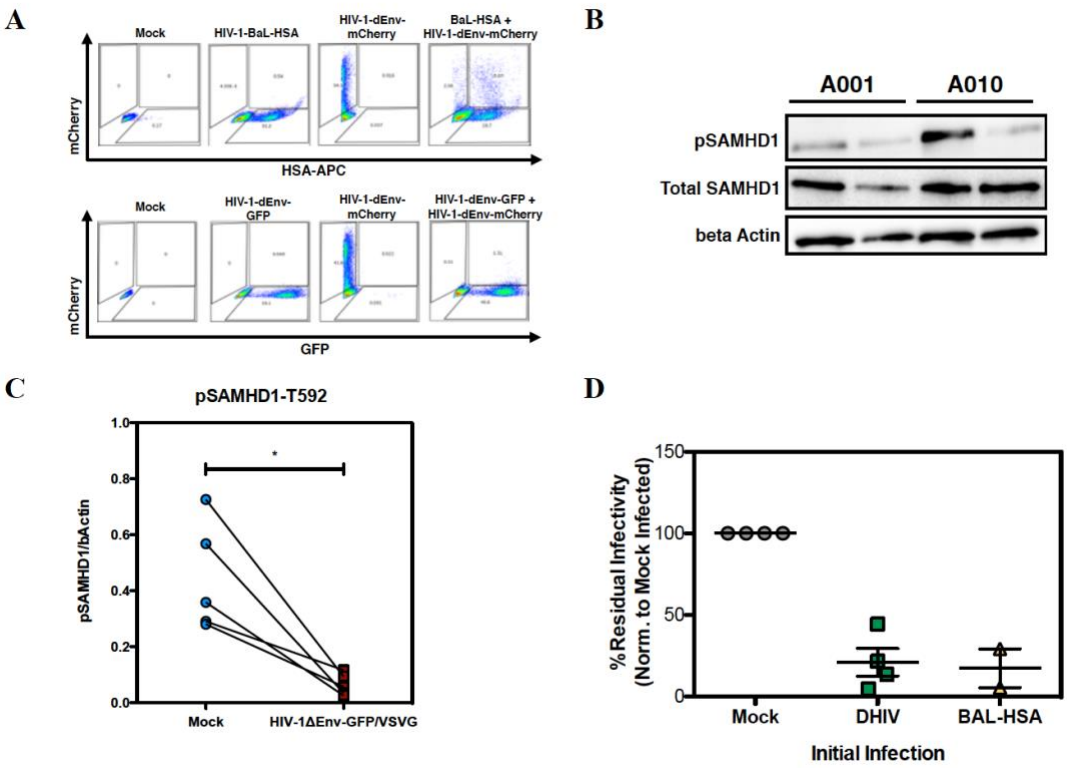

Figure 4.1. HIV-1 infection leads to SAMHD1 activation and protects macrophages from subsequent challenge. (A) Representative flow plots of MDM infected with HIV-1ΔEnv-GFP/VSVG or HIV-1-BAL-HSA, incubated for 48 hours, then challenged with HIV-1ΔEnv-mCherry/VSVG. (B) Western Blot analysis of MDM cell lysates from two donors (A001 and A010) in the presence or absence of HIV-1ΔEnv-GFP/VSVG. (C) Analysis of Western Blot via densitometry of five donors +/- HIV-1ΔEnv-GFP/VSVG. (D) Analysis of secondary infection of MDM with HIV-1ΔEnv-mCherry/VSVG following primary challenge with HIV-1ΔEnv-GFP/VSVG or HIV-1-BAL-HSA. Values represented as % secondary infection of primary mock-treated cells.

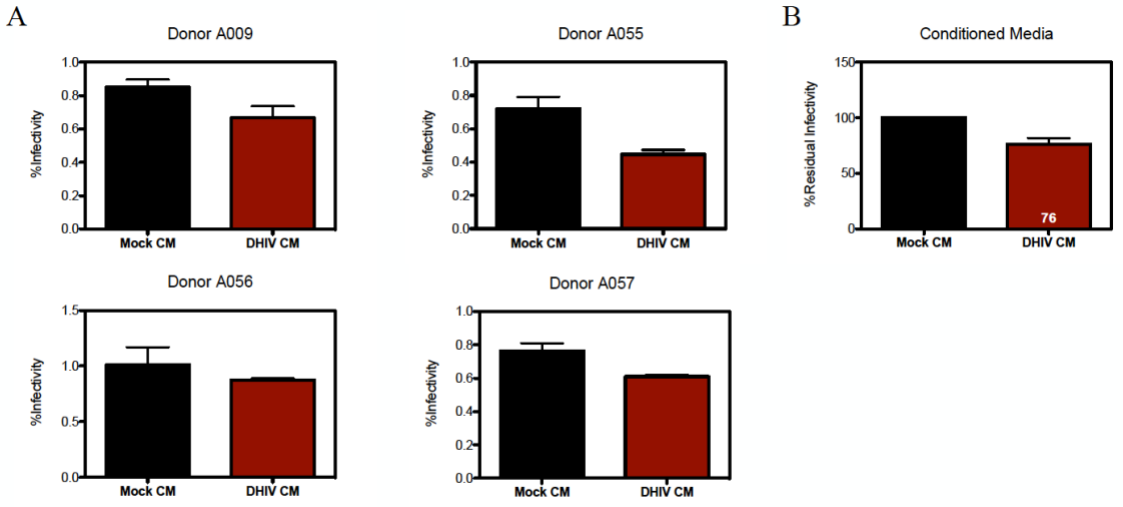

Figure 4.2. Conditioned media from infected macrophages fails to induce potent anti-HIV-1 activity in 'bystander' MDM. (A) Analysis of HIV-1ΔEnv-mCherry/VSVG infection in four donor (A009, A055, A056, A057) MDM infected following culture for 24 hours in media derived from mock-infected macrophages (black) or macrophages infected with HIV-1ΔEnv-GFP/VSVG (red). (B) Summary of % infectivity in MDM treated with HIV-1ΔEnv-GFP/VSVG-conditioned media. Values represent infection normalized to infection in MDM treated with mock-infected conditioned media (100%).

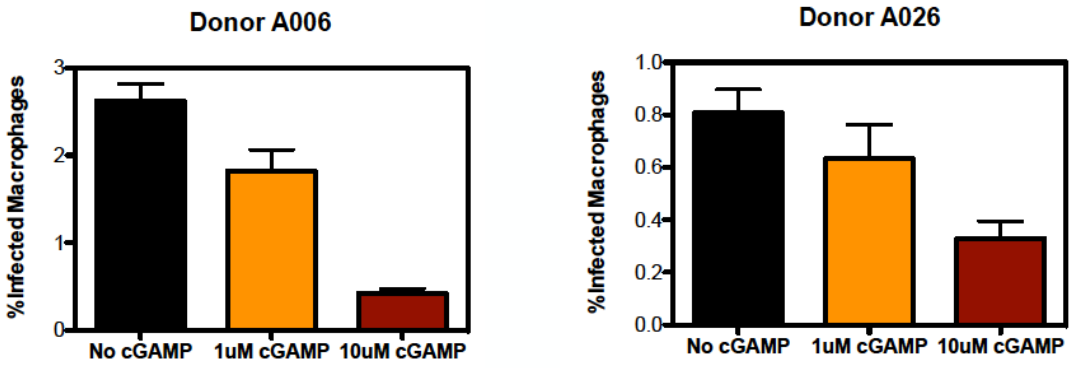

Figure 4.3. Exogenous cGAMP leads to HIV-1 restriction in MDM. Analysis of % infected macrophages following treatment with cGAMP (1micromolar, orange; 10micromolar, black).

Table 4.1. Phosphoproteomic analysis of HIV-1-infected macrophages.

Protein Target Name	Phospho Site (Human)	Full Target Protein Name	%CFC (A010 HIV-1 from A010 Mock)	Best Leads
CDK1/2	Y15	Cyclin-dependent protein-serine kinase 1/2 (CDC2)	558	Priority
p38d MAPK	Y182	Mitogen-activated protein-serine kinase p38 delta (MAPK13)	325	Priority
EGFR	Y1172	Epidermal growth factor receptor-tyrosine kinase (ErbB1)	190	Priority
p38g MAPK	Pan-specific	Mitogen-activated protein-serine kinase p38 gamma, ((MAPK12, ERK6)	151	Priority
IRAK1	Pan-specific	Interleukin 1 receptor-associated kinase 1 (Pelle-like protein kinase)	138	Priority
SIK3 (QSK)	Pan-specific	Serine/threonine-protein kinase SIK3	136	Priority
Tau	S721	Microtubule-associated protein tau	122	Priority
ELK1	Pan-specific	ETS domain-containing protein Elk-1	120	Priority
p38g MAPK	Pan-specific	Mitogen-activated protein-serine kinase p38 gamma, ((MAPK12, ERK6)	110	Priority
ALK	Pan-specific	Anaplastic lymphoma kinase	110	Priority
AKT2 (PKBb)	Pan-specific	RAC-beta serine/threonine-protein kinase	106	Priority
CDK4	Pan-specific	Cyclin-dependent protein-serine kinase 4	98	Priority
ZAP70	Pan-specific	Zeta-chain (TCR) associated protein-tyrosine kinase, 70 kDa	91	Priority
PKCm	S205	Protein-serine kinase C mu (Protein kinase D) (PRKD1, PKD1, PRKCM)	87	Priority
CHK1	Pan-specific	Checkpoint protein-serine kinase 1 (CHEK1)	77	Priority
SLK	S189	STE20-like serine/threonine-protein kinase (STK2)	75	Possible
MEK5	Pan-specific	MAPK/ERK protein-serine kinase 5 (MKK5, MAP2K5)	73	Possible
MEK3	Pan-specific	MAPK/ERK protein-serine kinase 3 beta isoform (MKK3 beta, MAP2K3)	70	Possible
CaMK1d	Pan-specific	Calcium/calmodulin-dependent protein-serine kinase 1 delta	69	Possible
OSR1	T185	Serine/threonine-protein kinase OSR1 (OXSR1)	67	Possible
CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	65	Possible
eIF4E	S209	Eukaryotic translation initiation factor 4 (mRNA cap binding protein)	63	Possible
NEK2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine kinase 2	62	Possible
MEKK1	Pan-specific	MAPK/ERK kinase kinase 1 (MAP3K1)	62	Possible
NEK2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine kinase 2	62	Possible
SRPK2	Y319	Serine/arginine-rich protein-specific kinase 2	61	Possible
PDGFRb	Pan-specific	Platelet-derived growth factor receptor kinase beta	61	Possible
CDK1	Pan-specific	Cyclin-dependent protein-serine kinase 1 (CDC2)	60	Possible
ASK1	Pan-specific	Apoptosis signal regulating protein-serine kinase 1 (MAP3K5)	59	Possible
p70 S6K	Pan-specific	Ribosomal protein S6 kinase beta-1 (RPS6KB1 p70S6Ka)	58	Possible
p38g MAPK	Pan-specific	Mitogen-activated protein-serine kinase p38 gamma, ((MAPK12, ERK6)	58	Possible
MYC	T58	Myc proto-oncogene protein	56	Possible
SRC	Pan-specific	Src proto-oncogene-encoded protein-tyrosine kinase	53	Possible
IKKa	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase alpha (CHUK, IkBKA)	51	Possible
PKCb	T500	Protein-serine kinase C beta 1 (PRKCB1)	-54	Possible

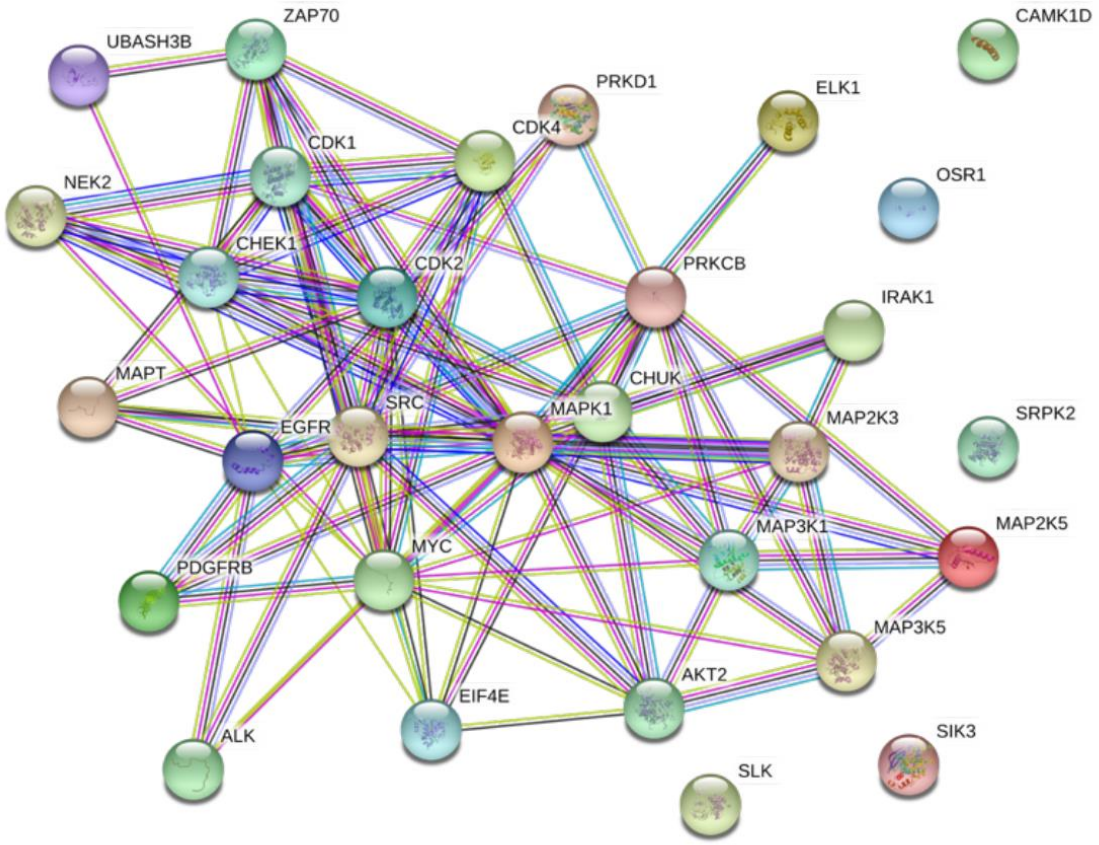

Figure 4.4. STRING protein-protein interaction map from phosphoproteomic hits. Genes identified as differentially expressed by phosphoproteomic analysis in HIV-1-infected cells were analyzed by STRING functional protein interaction networks v9.1.

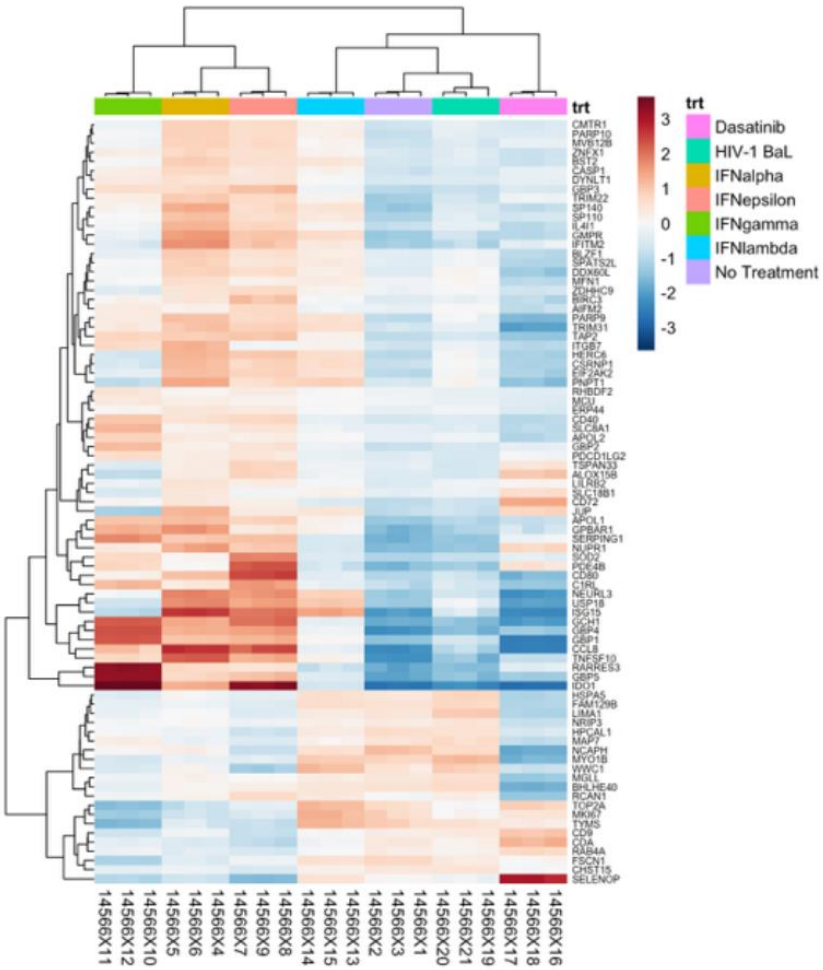

Figure 4.5. RNAseq analysis of 83 genes differentially regulated in all comparisons. rlog differences were hierarchically clustered, and values in the heatmap represent the amount a gene deviates in a specific sample from the gene's average across all samples.

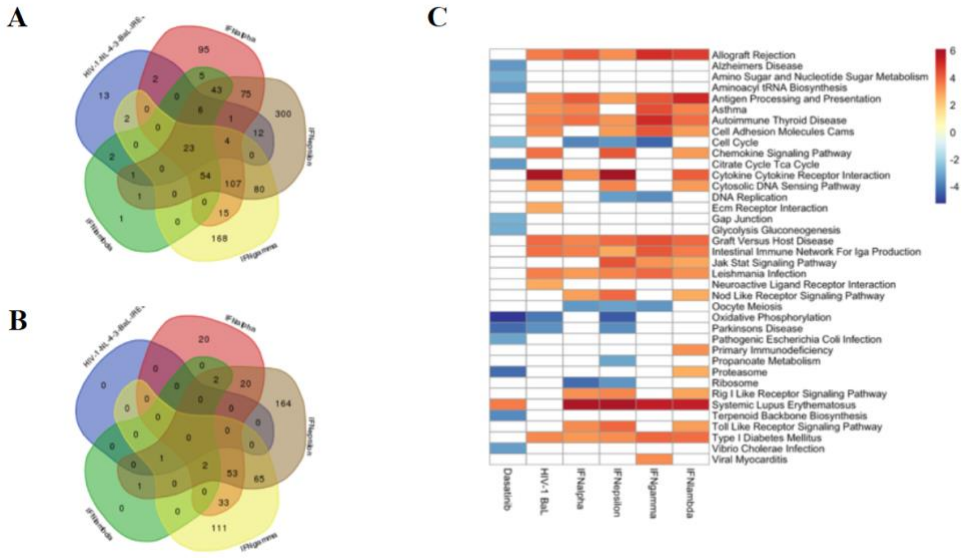

Figure 4.6. Comparison of type I, II, and III ISGs and HIV-1-infected MDM. (A) Comparison of RNAseq signatures exhibiting a log2 fold-change +2.0 or greater. (B) Comparison of RNAseq signatures exhibiting a log2 fold-change -2.0 or less. (C) Pathway analysis to determine positive (red) and negative (blue) enrichment of gene sets involved in the identified pathways across treatment groups.

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CHAPTER 5

TISSUE CULTURE METHODS FOR INVESTIGATING HIV-1 INFECTION IN MONOCYTE-DERIVED MACROPHAGES

Isolation of CD14+ monocytes from peripheral blood

1. In a sterile fume hood, prepare two 60mL syringes (BD, Catalog #309653) for peripheral phlebotomy by drawing 5mL ACD Formula A (Boston Bio Products, Catalog #IBB-400) into each syringe.
2. Cap each tube with syringe tip caps (BD, catalog #305819).
3. In a sterile fume hood, prepare four 50mL conical centrifuge tubes (Fisher Scientific, catalog #14-432-22 or similar) for peripheral blood mononuclear cell (PBMC) isolation by dispensing 15mL Lymphoprep density gradient medium (Stemcell Technologies, catalog #07581). Prepare two tubes for each 60mL syringe of whole blood drawn.
4. Take 60mL capped syringes to the phlebotomy station and perform healthy donor venous blood draw, filling as few as two syringes (120mL total) or as many as three syringes (180mL total) depending on cell quantities required. *Note: isolation of PBMC from 120mL of whole blood and subsequent CD14+ cell isolation will yield, on average, 20-40 million monocytes; isolation of PBMC from 180mL of whole blood and subsequent CD14+ cell isolation will yield, on average, 30-50 million monocytes. Cell counts vary widely based on the specific donor used.*
5. Working quickly, take whole blood into a sterile fume hood and slowly dispense 30mL of whole blood directly from the BD syringe into each centrifuge tube, without dilution, prepared in Step 3. *Note: allowing blood to sit for long periods of time, even with rocking at room temperature, will result in a diffuse PBMC layer following centrifugation over density gradient.*
6. Cap tubes containing Lymphoprep + whole blood. Balance tubes and place in

centrifuge, spinning at 1500rpm for 30 minutes at room temperature with 'acceleration' and 'deceleration' both set to 0 (~23-25 degrees Celsius). *Note: use of centrifuge brake will result in mixing of PBMC layer.*

7. Remove tubes from centrifuge. A distinct buffy coat (white cell layer) will be obvious under a clear layer of plasma. Remove 5-10mL of the plasma layer via aspiration, careful not to disrupt the PBMC buffy coat resting atop a layer of Lymphoprep.
8. Remove the buffy coat layer containing PBMCs and pipette into fresh 50mL conical tubes. Multiple tubes may be required.
9. Wash PBMCs with 1X PBS to generate a white cell pellet. Aspirate the supernatant without disturbing the pellet.
10. Resuspend PBMCs and transfer to a single 50mL conical centrifuge tube. Fill tube to 50mL total volume using MACS isolation buffer (1X PBS pH 7.2 with 0.5% Bovine Serum Albumin (BSA) and 2mM EDTA).
11. Remove a 10uL aliquot from the 50mL cell suspension and count.
12. Resuspend PBMCs in the appropriate volume of MACS isolation buffer, according to the manufacturer's recommendation (Miltenyi Biotec, catalog #130-050-201). *Note: use 8uL of buffer per 1 million cells.*
13. Add the appropriate volume of CD14+ magnetic beads, according to the manufacturer's recommendation (Miltenyi Biotec, catalog #130-050-201). *Note: use 2uL of beads per 1 million cells.*
14. Place cell suspension + beads at 4 degress Celsius for 15 minutes.
15. Wash cells in 10mL MACS isolation buffer from Step 10.

16. Isolate CD14+ cells using either the AutoMACS (program: Possel_s) or MACS LS Columns (Miltenyi Biotec, catalog #130-042-401).
17. Collect the CD14+ (positive) fraction, resuspend in 15mL Serum Free Media (SFM, RPMI 1640 supplemented with penicillin/streptomycin and L-glutamine). *Note: DO NOT USE SERUM-CONTAINING MEDIUM AS THIS INTERFERES WITH EFFICIENT CELL ADHERENCE.*
18. Remove a 10uL aliquot and count cells twice to ensure accurate measurements. Spin down CD14+ fraction and resuspend at a final concentration of 1 million cells per mL. This may require transfer to a 50mL conical centrifuge tube, depending on cell counts. *Note: DO NOT USE SERUM-CONTAINING MEDIUM AS THIS INTERFERES WITH EFFICIENT CELL ADHERENCE.*
19. Vigorously resuspend the cell pellet with a 1000uL pipette. Add SFM and vortex vigorously for 30 seconds in 5-10-second intervals. *Note: Monocytes will adhere to each other and to plastic if left stagnant for prolonged periods of time.*
20. Plate the monocytes (resuspended in SFM) by pipetting 600uL (600,000 cells/well, 24-well plates), 1200uL (1.2 million cells/well, 12-well plates), or 2400uL (2.4 million cells/well, 6-well plates) of cell suspension directly into each well. Swirl plates to ensure even cell distribution.
21. Place cells in an incubator for 2 hours to allow proper adherence. Note: Cells will begin to flatten as they adhere and will refract light differently. This step is important, as adding macrophage medium (serum-containing) too early will encourage detachment from the plate.
22. Once cells are adhering, generate macrophage growth medium in 50mL conical

centrifuge tubes by supplementing SFM from Step 17 with 12% pooled human serum (Innovative Research, catalog #IPLA-SER). *Note: Do not prepare macrophage growth medium in quantities exceeding 50mL, and always freeze down pooled human serum in 15mL aliquots for long-term storage.*

23. Following monocyte attachment, replace SFM with 600uL macrophage growth medium prepared in Step 22. Do not culture in any volume less than 600uL (24-well plates). Scale up accordingly for larger plates. *Note: DO NOT ASPIRATE SUPERNATANT FROM PLATES AS THIS WILL CAUSE THE CELLS TO DESSICATE. Gently pipette medium away by tilting the plate forward and placing the tip of the 1000uL pipette at the corner of the well. Work quickly and only replace media in 6 wells at a time to prevent desiccation.*

Culture and generation of monocyte-derived macrophages (MDM)

1. Following CD14+ isolation, plating, and media change to macrophage growth medium, place plated monocytes in an incubator for 5 days. Monitor macrophage differentiation, noting any overt detachment or plating inconsistencies. *Note: Cell confluency will affect downstream applications and yield inconsistent results if there is overt well-to-well variation.*
2. Carefully replace medium with fresh macrophage growth medium on day 5, being certain not to disturb the cell monolayer or allowing cells to dessicate.
3. On day 6-7, macrophages are ready for downstream applications including inhibitor studies and infection.

Generation of env-deleted HIV-1 stocks

1. Plate HEK293 FT cells in T-175 tissue culture flasks (Thermo Fisher, catalog #159910 or similar). Add 22mL DMEM supplemented with penicillin/streptomycin, L-glutamine, and 10% Fetal Bovine Serum (FBS).
2. Cells are ready to transfect when they have reached 70-80% confluency. Replace media with prewarmed complete DMEM 2 hours prior to transfection. *Note: Using prewarmed media will prevent cell detachment.*
3. For each plate, calculate the amount of plasmid required for 20μg of viral vector (HIV-1−ΔEnv-GFP or HIV-1−ΔEnv-mCherry) and 5μg of envelope (VSVG).
4. For each plate, mix 20μg of viral vector and 5μg of envelope in 900uL ddH_2O.
5. After diluting the plasmids, add 22μL of Chloroquine to each plate and mix gently.
6. Add 100μL of $CaCl_2$ to the 900μL diluted plasmid mix and mix by gentle pipetting.
7. Quickly add 1000μL of 2X HBS (Fisher Scientific, catalog #AAJ62623AK) to the diluted plasmid + $CaCl_2$ solution and mix thoroughly by pipetting. You will have a final volume of 2mL of precipitated plasmid solution for each plate.
8. Allow the mixture to rest at room temperature for 1 minute.
9. Gently pipette 2mL of the precipitated plasmid solution from Step 7 into each plate. Scale up accordingly for transfection of multiple plates.
10. Gently rock the plate containing 22mL media + Chloroquine + precipitated plasmid solution. *Note: Precipitated DNA should be visible by light microscopy and generally predicts a successful transfection.*

11. Place in the incubator and allow transfection to proceed for 6-8 hours. *Note: Overnight transfection may enhance transfection efficiency but may lead to cytotoxicity.*
12. Following 6-8 hour transfection, gently remove media and replace with fresh, pre-warmed DMEM.
13. For VSVG-pseudotyped viruses, collection can begin as early as 12 hours following completion of Step 12. Typically, viruses are collected at 24 and 48 hours posttransfection, or until cells become detached and appear dead.
14. Combine virus supernatants collected at 24 and 48 hours posttransfection and DNase (Sigma Aldrich, catalog #D5025) treat for 1 hour at 37 degrees Celsius to remove contaminating DNA plasmid. *Note: This step is optional for most applications but required for experiments involving PCR reactions relevant to the study of HIV-1.*
15. Optional: Concentrate VSVG pseudotyped virus by ultracentrifugation for 2 hours at 25,000rpm. Note: This is not required for macrophage infection as virus stocks produced using this protocol are sufficiently concentrated to generate maximum baseline infection in macrophages.
16. Aliquot virus stocks in Cryovials (Corning, catalog #09-761-71) for storage at -80 degrees Celsius, saving 50μL for p24 determination via ELISA (ZeptoMetrix, catalog #0801111). Note: It is best to save multiple virus supernatants to run a single p24 ELISA.

Generation of replication-competent HIV-1 stocks

1. Plate HEK293 FT cells in T-175 tissue culture flasks (Thermo Fisher, catalog #159910 or similar). Add 22mL DMEM supplemented with penicillin/streptomycin, L-glutamine, and 10% Fetal Bovine Serum (FBS).
2. Cells are ready to transfect when they have reached 70-80% confluency. Replace media with prewarmed complete DMEM 2 hours prior to transfection. *Note: Using prewarmed media will prevent cell detachment.*
3. For each plate, calculate the amount of plasmid required for 25μg of viral vector (HIV-1-NL-4-3-AD8 or HIV-1-NL-4-3-BaL-IRES-HSA).
4. For each plate, dilute 25μg viral vector in 900uL ddH_2O. *Note: HIV-1-NL-4-3-AD8 and HIV-1-NL-4-3-BaL-IRES-HSA maintain envelope intact and thus do not require complementation with an envelope-encoding plasmid.*
5. After diluting the plasmids, add 22μL of Chloroquine to each plate and mix gently.
6. Add 100μL of $CaCl_2$ to the 900μL diluted plasmid mix and mix by gentle pipetting.
7. Quickly add 1000μL of 2X HBS (Fisher Scientific, catalog #AAJ62623AK) to the diluted plasmid + $CaCl_2$ solution and mix thoroughly by pipetting. You will have a final volume of 2mL of precipitated plasmid solution for each plate.
8. Allow the mixture to rest at room temperature for 1 minute.
9. Gently pipette 2mL of the precipitated plasmid solution from Step 7 into each plate. Scale up accordingly for transfection of multiple plates.
10. Gently rock the plate containing 22mL media + Chloroquine + precipitated

plasmid solution. *Note: Precipitated DNA should be visible by light microscopy and generally predicts a successful transfection.*

11. Place in the incubator and allow transfection to proceed for 6-8 hours. *Note: Overnight transfection may enhance transfection efficiency but may lead to cytotoxicity.*
12. Following 6-8-hour transfection, gently remove media and replace with fresh, pre-warmed DMEM.
13. Collect viral supernatants at 24 and 48 hours posttransfection, or until cells become detached and appear dead.
14. Combine virus supernatants collected at 24 and 48 hours posttransfection and DNase (Sigma Aldrich, catalog #D5025) treat for 1 hour at 37 degrees Celsius to remove contaminating DNA plasmid. *Note: This step is optional for most applications but required for experiments involving PCR reactions relevant to the study of HIV-1.*
15. Aliquot virus stocks in Cryovials (Corning, catalog #09-761-71) for storage at -80 degrees Celsius, saving 50μL for p24 determination via ELISA (ZeptoMetrix, catalog #0801111). *Note: It is best to save multiple virus supernatants to run a single p24 ELISA.*

Infection of MDM in static culture

1. Culture macrophages for 7 days according to the protocol described in "Culture and Generation of Monocyte-Derived Macrophages (MDM) from Healthy Donor CD14+ Monocytes."

2. On day 7, thaw virus from Cryovials at room temperature. Calculate the volume of viral supernatants equivalent to 500ng of p24 as determined by ELISA in "Generation of HIV-1-NL-4-3-AD8 and HIV-1-NL-4-3-BaL-IRES-HSA virus stocks for infection of MDM in static culture."
3. Mix the appropriate amount of virus with macrophage growth medium such that each infection contains 500ng of p24 in a 250μL total final volume.
4. Gently remove macrophage growth media from each well using a 1000μL pipette by tilting the plate and pipetting. Work quickly and refrain from pulling media from more than 6 wells at a time to prevent desiccation.
5. Gently pipette the 250μL virus mixture against the side wall of each individual well, allowing the solution to cascade gently over the cells. *Note: Do not pipette the virus solution directly onto the macrophage monolayer, as disturbances can vastly affect the viability, infectivity, and behavior of MDM.*
6. Place the culture plates back in the incubator for 4-6 hours to allow infection to proceed. *Note: DO NOT leave macrophages in low-volume (250μL) culture overnight. Evaporation will occur and expose macrophages to desiccation.*
7. Gently remove virus supernatant following 4-6-hour incubation, wash once with 250μL macrophage growth medium to remove any unbound virus, and replace with 600μL fresh macrophage growth medium. Analyze 48 hours later.

Analysis of MDM infection

1. Remove medium 48 hours postinfection and replace with 400μL Gibco StemPro Accutase Cell Dissociation Reagent (Fisher Scientific, catalog #A1110501).

Allow macrophages to detach in Accutase for 2-3 hours. Depending on confluency and cell density, detachment may take as long as 4 hours. *Note: Cells will require gentle washing to aid in detachment. This is best accomplished by gently taking up Accutase using the 1000μL pipette and slowly washing around each well until the majority of cells are in suspension.*

2. Once cells are detached, place in tubes suitable for flow cytometry and wash with 1 volume 1X PBS.
3. Prior to staining for analysis of cell surface markers, F_c receptors present on macrophages must be blocked using FcR Blocking Reagent, Human (Miltenyi Biotec, catalog #130-059-901) and stained for viability using eBioscience Fixable Viability Dye eFluor450 (Fisher Scientific, catalog #65-0863-14). For each test, dilute 0.5μL of Viability Dye and 2μL of FcR Blocking Reagent in 100μL of 1X PBS. Incubate at 4 degrees Celsius for 30 minutes.
4. Stain for any pertinent cell surface markers according to the manufacturer's protocol. Wash unbound antibody before proceeding to Step 5. *Note: Accutase treatment may prevent detection of certain epitopes by flow cytometry – refer to the manufacturer for details.*
5. For infection with HIV-1−ΔEnv-GFP/VSVG and HIV-1−ΔEnv-mCherry/VSVG, fix cells using BD Cytofix (Becton Dickinson, catalog #554714) according the manufacturer's protocol. For infection with HIV-1-NL-4-3-AD8 or HIV-1-NL-4-3-BaL-IRES-HSA, proceed to Step 7.
6. Wash cells by adding 1mL 1X PBS. Aspirate all but 200μL of the wash solution. Cells are now ready for flow cytometry.

7. Fix and permeabilize cells from Step 4 using BD Cytofix/Cytoperm (Becton Dickinson, catalog #554722) according to the manufacturer's protocol.
8. Dilute BD Perm/Wash (Becton Dickinson, catalog #554723) according to the manufacturer's protocol.
9. Wash cells using BD Perm/Wash according to the manufacturer's protocol.
10. For each test, dilute 1μL of anti-HIV-1 core antigen, KC57 (Fisher Scientific, catalog #CO6604665) in 100μL diluted BD Perm/Wash solution. Allow p24 stain to proceed for 30 minutes at 4 degrees Celsius, protected from light. *Note: You must use BD Perm/Wash to maintain permeabilization during the intracellular staining process.*
11. After 30 minutes, wash cells in 1X BD Perm/Wash, aspirating all but 200μL following centrifugation. Cells are ready for flow cytometry.

Whole cell lysate extraction and Western Blot analysis

1. Culture macrophages for 6-7 days according to the protocol described in "Culture and Generation of Monocyte-Derived Macrophages (MDM) from Healthy Donor CD14+ Monocytes."
2. Prepare 10mL of NETN containing one tablet each cOmplete ULTRA EDTA-Free Protease Inhibitor Cocktail Tablet, mini (Sigma Aldrich, catalog #5892953001) and PhosSTOP (Sigma Aldrich, catalog #4906845001). Mix thoroughly at room temperature until tablets have completely dissolved, then aliquot into 1.5mL Eppendorf tubes and freeze at -20 degrees Celsius.
3. Remove macrophage growth medium from each well and wash cells with 500μL

1X PBS. While NETN is cold, add 100μL per well and rock the plate to ensure all cells are covered.

4. Allow lysis to occur for 1 minute, then using the 200μL pipette, begin to wash the cells/cell debris off the bottom of the well.
5. Once all cells have been released from the well, place the NETN cell lysate in a fresh 1.5mL Eppendorf and place on ice for 30 minutes.
6. Take lysates on ice to a sonicator bath and pulse lysates once for 15 seconds to shear DNA and clarify extracts. Note: Allowing lysates to warm to room temp or longer sonication will affect quality of Western Blots, specifically when probing for phosphoproteins.
7. After sonication, spin lysates to pellet cell debris. Carefully remove the aqueous layer and place in a fresh 1.5mL Eppendorf. Label Eppendorf with donor ID, cell type, any experimental conditions used, and the date.
8. Place extracts at -20 degrees Celsius for long-term storage or proceed to Step 8 for quantification by BCA.
9. Dilute extracts 1:10 in NETN, generating a 60μL final volume. Note: You do NOT need to use NETN supplemented with PhosSTOP and cOmplete Protease Inhibitor Cocktail for protein quantification by BCA.
10. Following the manufacturer's protocol, perform the Pierce BCA Protein Assay using the diluted cell extracts from Step 9 (Thermo Fisher, catalog #23225).
11. Incubate for 30 minutes at 37 degrees Celsius. After 30 minutes, allow the plate to cool to room temperature prior to analyzing on the plate reader.
12. Read the absorbance on the plate reader with the spectrophotometer set to 562nm.

13. Calculate the final concentration of the extracts by multiplying the value read on the plate reader by 10.
14. For Western Blots, calculate the volume required for 12.5μg of protein. Dilute to 25μL in NETN containing PhosSTOP and cOmplete ULTRA tablets. *Note: It is crucial to dilute in NETN with phosphatase and protease inhibitors at this step.*
15. Add 4.2μL 6X Lamelli Buffer and boil at 100 degrees Celsius for 10 minutes. Note: Use lid locks to prevent caps from opening during boiling.
16. Spin 1.5mL Eppendorf tubes down to collect the 29.2μL final total volume.
17. Pipette 26μL of the protein/Lamelli Buffer mixture into each well of the precast Western Blot gel.
18. Allow electrophoresis to run at 70-90V until the dye has run to the end of the gel. *Note: Running at lower voltage prevents irregularities ('smiles') in the gel.*
19. While the gel is running, prepare a section of membrane cut to mirror the size and shape of the blot paper (Bio-Rad, catalog #1703932).
20. Activate the membrane by rocking in pure Methanol for 5-10 minutes.
21. Prepare a solution containing 10mL 10X Transfer Buffer, 20mL Methanol, and 70mL ddH_2O (total volume = 100mL) for each gel run. Place the membrane in this solution and rock for 20-30 minutes to equilibrate the membrane.
22. As the gel is nearing its end point, soak a piece of blot paper in the transfer buffer solution from Step 21 and place on the transfer station. Roll the blot paper firmly enough to remove air bubbles, but be careful not to squeeze all of the transfer solution out.
23. When gel has finished running, remove from electrophoresis tank and quickly

rinse in water.

24. Take gel to the transfer station and crack the sides of the gel casing and remove the top portion. *Note: You must work quickly to prevent the gel from desiccating and to maintain the appropriate moisture content on the blot paper to ensure efficient protein transfer.*
25. Remove the membrane from the transfer buffer from Step 21 and gently place on the wet blot paper. Pour a small amount of the transfer solution on top of the membrane to ensure it does not dry out.
26. With a small amount of pressure, roll any air bubbles out from between the blot paper and the membrane.
27. Working quickly, place the gel on the membrane by handling at the thickest part of the gel (the bottom). Do your best to ensure there are no large air bubbles underneath the gel.
28. Soak another piece of blot paper in the transfer buffer solution and quickly place atop the gel. Roll the entire sandwich to ensure all air bubbles are removed and the blot paper is snug against the gel.
29. Set the transfer station to run at 20V, 0.4A, for 30 minutes.
30. Once the transfer is complete, place the membrane in 4% BSA (prepared in TPBS, NOT water) and block for at least 1 hour.
31. Cut the membrane after blocking into the desired sections and incubate overnight, rocking at 4 degrees C with the desired primary antibody diluted according to the manufacturer's instructions.
32. The next day, remove the primary antibody and wash 3X for 5 minutes in TPBS.

33. Add the secondary antibody, diluted in 4% BSA, and incubate rocking for at least 2 hours at room temperature. *Note: Actin is ready to be developed after 30 minutes of rocking with the secondary antibody.*
34. Wash the membranes 3X for 5 minutes in TPBS.
35. Develop each membrane individually by rocking for 3.5 minutes in SuperSignal West Pico PLUS Substrate (Thermo Fisher, catalog #345850).

Generation of Vpx-VLPs and degradation of SAMHD1

1. Plate HEK293 FT cells in T-175 tissue culture flasks (Thermo Fisher, catalog #159910 or similar). Add 22mL DMEM supplemented with penicillin/streptomycin, L-glutamine, and 10% Fetal Bovine Serum (FBS).
2. Cells are ready to transfect when they have reached 70-80% confluency. Replace media with prewarmed complete DMEM 2 hours prior to transfection. *Note: Using prewarmed media will prevent cell detachment.*
3. For each plate, calculate the amount of plasmid required for 20μg of viral vector (SIV3+ for Vpx(+)VLPs or SIV3+ΔVpx for control Vpx(-)VLPs) and 5μg of envelope (VSVG).
4. For each plate, mix 20μg of viral vector and 5μg of envelope in 900uL ddH_2O.
5. After diluting the plasmids, add 22μL of Chloroquine to each plate and mix gently.
6. Add 100μL of $CaCl_2$ to the 900μL diluted plasmid mix and mix by gentle pipetting.
7. Quickly add 1000μL of 2X HBS (Fisher Scientific, catalog #AAJ62623AK) to

the diluted plasmid + $CaCl_2$ solution and mix thoroughly by pipetting. You will have a final volume of 2mL of precipitated plasmid solution for each plate.

8. Allow the mixture to rest at room temperature for 1 minute.
9. Gently pipette 2mL of the precipitated plasmid solution from Step 7 into each plate. Scale up accordingly for transfection of multiple plates.
10. Gently rock the plate containing 22mL media + Chloroquine + precipitated plasmid solution. *Note: Precipitated DNA should be visible by light microscopy and generally predicts a successful transfection.*
11. Place in the incubator and allow transfection to proceed for 6-8 hours. *Note: Overnight transfection may enhance transfection efficiency but may lead to cytotoxicity.*
12. Following 6-8-hour transfection, gently remove media and replace with fresh, pre-warmed DMEM.
13. For VSVG-pseudotyped VLPs, collection can begin as early as 12 hours following completion of Step 12. Typically, viruses are collected at 24 and 48 hours posttransfection, or until cells become detached and appear dead.
14. Combine virus supernatants collected at 24 and 48 hours posttransfection and DNase (Sigma Aldrich, catalog #D5025) treat for 1 hour at 37 degrees Celsius to remove contaminating DNA plasmid. *Note: This step is optional for most applications but required for experiments involving PCR reactions relevant to the study of HIV-1.*
15. Concentrate VSVG pseudotyped VLPs by ultracentrifugation for 2 hours at 25,000rpm.

16. Aspirate the supernatant down to 2-3mL, careful not to remove any of the pelleted VLPs. Resuspend the pellets by gentle pipetting in a total volume of 5mL per tube.
17. Combine each resuspension and aliquot virus stocks in Cryovials (Corning, catalog #09-761-71) for storage at -80 degrees Celsius, saving 50μL for ZeptoMetrix p27 determination via ELISA (Fisher Scientific, catalog #22-156-775). Note: It is best to save multiple virus supernatants to run a single p27 ELISA.

siRNA delivery to MDM

1. ON-TARGETplus SMARTpool siRNAs are ordered from Dharmacon and diluted to achieve a 20μM stock. For 5nmol of siRNA, resuspend in 250μL 1X siRNA buffer. Nanodrop to confirm concentration according to the manufacturer's specifications.
2. Aliquot siRNAs to 25μL stocks to avoid repeated freeze-thaw cycles.
3. Begin siRNA experiments when macrophages have reached day 5.
4. Determine the number of wells required for the experiment. Note: Typically, 6 wells will be needed – 3 for infections (triplicate) and 3 for generation of whole cell lysate (combined).
5. Prewarm OptiMEM media in the 37 degree bed bath.
6. For each well to be treated, aliquot 2.5μL of the 20μM stock siRNA and dilute in 47.5μL warmed OptiMEM. *Note: For 6 wells, prepare 6.5 'reactions'. This is the equivalent of 16.25μL stock siRNA + 308.75μL OptiMEM.*

7. For each well to be treated, aliquot 2.5μL Lipofectamine RNAiMAX (Fisher Scientific, catalog #13778150) and dilute in 47.5μL warmed OptiMEM. *Note: For 6 wells, prepare 6.5 'reactions'. This is the equivalent of 16.25μL Lipofectamine RNAiMAX + 308.75μL OptiMEM (325μL of diluted Lipofectamine for each siRNA to be used).*
8. Combine the diluted siRNA with the diluted Lipofectamine RNAiMAX for a final volume of 650μL. This is enough siRNA for 6 wells at 100μL per well.
9. Allow the mixture to rest at room temperature for 10 minutes.
10. After 10-minute incubation, add 900μL warmed OptiMEM for a final volume of 1550μL.
11. Aliquot 225μL of the mixture from Step 10 to each well.
12. Incubate for 6 hours at 37 degrees Celsius. Note: Longer incubation times will allow for evaporation, exposing cells to desiccation.
13. After 6 hours incubation, add 225μL macrophage growth medium and incubate overnight at 37 degrees Celsius. *Note: The concentration of siRNA is now 111nM.*
14. The next morning (day 6 postdifferentiation), repeat Steps 4-13.
15. On day 7, cells are ready for downstream applications.